This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07H

A2

(11) International Publication Number: **WO 94/00463**

(43) International Publication Date: 6 January 1994 (06.01.94)

(21) International Application Number: PCT/EP93/01575

(22) International Filing Date: 21 June 1993 (21.06.93)

(30) Priority data:
PD92A000110 19 June 1992 (19.06.92) IT

(71) Applicant *(for all designated States except US)*: M.U.R.S.T. ITALIAN MINISTRY FOR UNIVERSITIES AND SCIENTIFIC AND TECHNOLOGICAL RESEARCH [IT/IT]; Lungotevere Thaon de Revel, 39, I-Rome (IT).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : PREHM, Peter [DE/DE]; In der Weede 75, D-48163 Münster (DE). O'REGAN, Michael [IT/IT]; Via Nazareth, 15, I-35100 Padova (IT). CALLEGARO, Lanfranco [IT/IT]; Via Bravi, 35, I-35020 Ponte di Brenta (IT). LELLIG, Sabine [DE/DE]; In der Weede 75, D-48163 Münster (DE).

(74) Agent: VOSSIUS & PARTNER (NO.31); Siebertstrasse 4, P.O. Box 86 07 67, D-8000 Munich 86 (DE).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: DNA ENCODING HYALURONIC ACID SYNTHASE (HAS), EXPRESSION VECTORS CONTAINING THIS GENE, AND PRODUCTION OF HYALURONIC ACID BY TRANSEFORMED MICROORGANISMS AND IN VITRO

(57) Abstract

An isolated DNA fragment comprising a nucleotide sequence encoding hyaluronic acid synthase is provided. Also provided are recombinant expression vectors comprising this sequence, capable of expressing hyaluronic acid synthase in prokaryotic and eukaryotic cells, as well as host cells harboring such expression vectors. Such cells are capable of producing hyaluronic acid synthase, hyaluronic acid, or both, which can be recovered from these cells, the culture medium, or both. Also provided are a method of producing hyaluronic acid synthase, methods of producing hyaluronic acid both *in vivo* and *in vitro*, and hyaluronic acid produced by these methods. In the case of *in vitro* production, the molecular weight of hyaluronic acid can be varied by varying the time of incubation of hyaluronic acid precursors in the presence of hyaluronic acid synthase and/or the reaction conditions. Lastly, a method of purifying hyaluronic acid synthase from cultured cells is also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic			SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	UA	Ukraine
DE	Germany	MG	Madagascar	US	United States of America
DK	Denmark	ML	Mali	UZ	Uzbekistan
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

DNA ENCODING HYALURONIC ACID SYNTHASE (HAS), EXPRESSION VECTORS CONTAINING THIS GENE, AND PRODUCTION OF HYALURONIC ACID BY TRANSFORMED MICROORGANISMS AND IN VITRO

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a DNA fragment encoding the hyaluronic acid synthase (HAS) of *Streptococcus equisimilis* D181, the nucleotide sequence of this fragment, and the industrial use of this DNA for the production of HAS and hyaluronic acid.

Description of Related Art

Hyaluronic acid (HA) is a linear glucosaminoglycan composed of repetitive disaccharide subunits linked together by β(1-4) bonds. Each subunit is formed by glucuronic acid bound by a β(1-3) bond to N-acetylglucosamine. A ubiquitous component of the extracellular matrix, HA has many important functions in humans and animals, for instance in cell lubrication, mobility and adhesion. It therefore plays an important role in cell-to-cell interactions, cell differentiation

and tissue repair. Owing to these and its other interesting properties, HA is widely used in the pharmaceutical and health care industries.

Until recently, this polymer was commonly obtained by purification from animal sources such as rooster combs. This source of raw material presents certain disadvantages such as the fact that it offers limited yields and because purification of the high molecular weight polymer is hampered by HA's tendency to form complexes with other molecules, including proteins. To overcome these problems, there have been efforts in recent years to investigate streptococci as an alternative source of HA.

HA can indeed be produced from the A and C groups of streptococci (Lancefield's classification), and it also seems to be a major virulence factor in infections caused by these microorganisms (Wessels et al., Proc. Natl. Acad. Sci. USA, 1991, 88: 8317-8321). The HA molecule produced from these bacteria is chemically identical to that obtained from animal tissues, and it is relatively easy to isolate polymers with molecular weights of about two million Daltons (Da) from culture media. It is a generally accepted fact that HA with even higher molecular weights can be used in a wide range of applications thanks to its high viscosity and biocompatibility.

SUMMARY OF THE INVENTION

The production of high purity HA of varying molecular weights for particular applications can be achieved by obtaining a more detailed understanding of the mechanism of synthesis of HA at the molecular level, and using this information to generate new HA polymers, thus broadening the scope of its existing applications and creating new ones.

Recently, much effort has been expended in studying the activity of the enzyme responsible for HA synthesis, hyaluronic acid synthase (HAS). HAS has been found to be associated with the plasma membrane in both mammalian and bacterial cells (Markovitz et al., J. Biol. Chem., 1962, 237: 273-279; P. Prehm, Biochem. J., 1984, 220: 597-600) where it acts as a catalyst for polymerization of the precursors UDP-glucuronic acid and UDP-N-acetylglucosamine to form the extracellular linear molecule of HA. Various attempts have been made to study HAS components in more detail by purifying the enzyme. Purification from mammalian cells has proved unsuccessful, thus focusing attention on bacterial systems. By using different techniques--isolation of the protein linked with the newly-formed HA, purification of an enzymatically active synthase, isolation of mutant strains of streptococci that do not produce HA--it has been shown that the HAS of streptococci consists of a membrane-associated protein of about 52 kDa which may form a complex with one or more proteins (Prehm et al., Biochem. J., 1986, 235: 887-889; Mausolf et al., Biochem. J., 1990, 267: 191-196). The activity of HAS in streptococcal membranes is blocked by antibodies directed against the 52 kDa protein (Prehm et al., Biochem. J., 1986, 235: 887-889), indicating that this polypeptide is HAS and constitutes an integral part of the possible complex for HA synthesis.

The identification of the protein component(s) involved in HA synthesis and the cloning of the corresponding gene(s) offers considerable potential. The cloning of the relevant gene(s) under the control of the natural promoter or an alternative strongly active promoter would allow the construction of recombinant bacterial strains which could be used either as more efficient producers of HA or as sources of purified HAS.

The ability to isolate sufficient quantities of highly purified active HAS would permit the development of an efficient in vitro synthetic method for HA production. HA produced by such a system would offer considerable advantages over the presently available products extracted from animal or bacterial sources. For example, using an immobilized enzyme system, production of HA of practically 100% purity would be possible. By using varying reaction times and/or conditions, the molecular weight of the resulting polymer could be controlled, thus allowing the synthesis of HA of particular molecular weights for specific applications. The possibility of using synchronized reaction conditions would permit the polydispersity of the molecular weight to be controlled, also thus resulting in a much more homogeneous product. Japanese Patent Application Laid-Open JP 90231093 A2 discloses an in vitro system for the preparation of HA. However, rather than employing purified HAS, streptococcal membranes are employed. The present invention discloses the cloning of an isolated, purified DNA fragment from *Streptococcus equisimilis* D181 that encodes the HAS protein of about 52 kDa, and the nucleotide sequence of the gene itself, thus allowing the manipulation of the genetics and enzymology of HA synthesis in eukaryotic and prokaryotic cells.

A procedure has been disclosed which allegedly allows the isolation of a DNA fragment from *Streptococcus equisimilis* D181 encoding hyaluronic acid synthase (PCT publication No. WO 91/03559). The scientific data contained therein are insufficient to prove that the DNA fragment isolated effectively encodes this enzyme, and do not permit those of skill in the art to repeat the manipulations to obtain the putative HA synthase-encoding clone.

Using recombinant DNA techniques, the present inventors constructed a genomic bank of *Streptococcus equisimilis* D181 DNA in λgt11. The clones encoding HAS were isolated using antibodies directed against the HAS protein of about 52 kDa. A clone expressing HAS containing a DNA fragment of about 3.1 kb was identified. This fragment was then subcloned to obtain a λgt11 clone containing an insert of 2.1 kb encoding HAS. The nucleotide sequence of this fragment was determined, and a region encoding a protein of 56 kDa (the molecular weight of which had previously been determined as 52 kDa on the basis of its electrophoretic behavior) was identified. Southern blotting data showed that the HAS gene is conserved in organisms that synthesize HA, and is absent from those that do not produce it.

Accordingly, it is an object of the present invention to provide an isolated DNA fragment, comprising a nucleotide sequence encoding hyaluronic acid synthase.

It is another object of the present invention to provide a recombinant expression vector, comprising a nucleotide sequence encoding hyaluronic acid synthase, wherein said vector is capable of expressing said hyaluronic acid synthase in a transformed prokaryotic or eukaryotic cell.

A further object of the present invention is to provide a prokaryotic or eukaryotic cell transformed with said recombinant expression vector, wherein said cell is capable of producing hyaluronic acid synthase, hyaluronic acid, or both.

Yet a further object of the present invention is to provide a method of producing hyaluronic acid synthase, comprising:

(a) providing a prokaryotic or eukaryotic cell harboring a DNA sequence encoding hyaluronic acid synthase, wherein said DNA sequence is expressible in said cell;

(b) culturing said cell in a culture medium; and

(c) recovering said hyaluronic acid synthase from said cell or from said culture medium.

Another object of the present invention to provide a method of producing hyaluronic acid, comprising:

(a) providing a recombinant prokaryotic or eukaryotic cell harboring a DNA sequence encoding hyaluronic acid synthase, wherein said DNA sequence is expressible in said cell;

(b) culturing said cell in a culture medium; and

(c) recovering said hyaluronic acid from said cell or from said culture medium.

A still further object of the present invention is to provide a method of purifying hyaluronic acid synthase, comprising:

(a) culturing cells expressing a gene encoding hyaluronic acid synthase;

(b) harvesting said cells of step (a);

(c) disrupting said cells under conditions that minimize the inactivation of hyaluronic acid synthase;

(d) isolating membranes of said cells;

(e) suspending said membranes in a buffer;

(f) extracting said hyaluronic acid synthase from said membranes by adding a detergent to the membrane suspension of step (e), and then centrifuging to produce a detergent phase containing said hyaluronic acid synthase;

(g) adding polyethylene glycol to said detergent phase of step (f), thereby forming an aqueous phase containing said hyaluronic acid synthase; and

(h) recovering said hyaluronic acid synthase from said aqueous phase of step (g).

A still further object of the present invention is to provide a method of producing hyaluronic acid of varying molecular weight, comprising:

(a) incubating UDP-GlcA and UDP-NAcGlc with hyaluronic acid synthase under conditions suitable for the synthesis of hyaluronic acid; and

(b) recovering hyaluronic acid,

wherein the molecular weight of said hyaluronic acid can be varied by varying the reaction time and/or reaction conditions under which said UDP-GlcA and said UDP-NAcGlc are incubated in the presence of said hyaluronic acid synthase.

A final object of the present invention is to provide hyaluronic acid produced by the foregoing method, wherein said hyaluronic acid is free of any contaminating proteins, pyrogenic or inflammatory substances, or viruses.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawings provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features, and advantages of the present invention will be better understood from the following detailed descriptions taken in conjunction with the accompanying drawings, all

of which are given by way of illustration only, and are not limitative of the present invention, in which:

Fig. 1 shows a comparison between HAS from streptococci and recombinant HAS. The HAS was isolated from the protoplastic membranes of *Streptococcus equisimilis* D181 (A), from *E.coli* lysates of λgt11/2LK (B), and λgt11 (C) (negative control). The proteins were analyzed by SDS-PAGE and Western blotting, using polyclonal antibodies directed against HAS.

Fig. 2 shows the strategy for sequencing the HAS gene. The DNA of the original λgt11/2LK clone was digested with the restriction enzyme *Eco*RI, and the insert of 2.1 kb containing the whole HAS gene was subcloned (λgt11/2LK-D, thick arrow). The extent and direction of each sequence is indicated by the fine arrows. The region encoding HAS is represented by the rectangle under the 2.1 kb fragment. The small rectangles underneath represent the segments verified by amino acid analysis of the HAS purified from streptococci.

Fig. 3 shows the nucleotide sequence of recombinant HAS and the deduced amino acid sequence. The nucleotides are numbered beside the sequence shown from 5′ to 3′. The deduced amino acid sequence is written under the nucleotide sequence; the residues determined from the peptide sequence are underlined. The probable promoter sequence at -35 and -10, marked by a line above the sequence, and the ribosome binding site (SD), are shown in the space above the encoding sequence. Palindromic sequences are shown by broken lines. The putative signal peptidase cleavage site is indicated by the arrow.

Fig. 4 shows the location of inverted sequences in the HAS gene. HAS contains two inverted repetitive sequences in its AT rich region before the encoding region and another two which, encompassing the complete coding region, could form a potential loop of 1653 bp.

Fig. 5 shows the hydrophobic profile of HAS. This graph was generated using Kyte and Doolittle's standard parameters (J. Mol. Biol. 1982, 157: 105-132). The y-axis indicates the hydrophobicity value of the amino acid residues starting from the initiation codon.

Fig. 6 shows the results of Southern blotting analysis.

A. Probe: PCR product of 3.1 kb derived from λgt11/2LK; Sample: 1, PCR product; 2, *Eco*RI digested λgt11/2LK; 3-6, *S. equisimilis* genomic DNA, *Eco*RI-, *Hind*III-, *Bam*HI-, and *Eco*RV-digested, respectively; *S. equi* genomic DNA, same enzymes; 11-14, *S. sanguis* genomic DNA, same enzymes; 15-18, *S. zooepidemicus* genomic DNA, same enzymes.

B. Probe: internal fragment *Xba*I-*Pst*I. Sample: 1, PCR product; 2, *Eco*RI digested λgt11/2LK; 3-6, *S. equi* genomic DNA, enzymes as in A; 7-10, *S. equisimilis* genomic DNA, enzymes as in A; 11-14, *S. zooepidemicus* genomic DNA, enzymes as in A.

C. Probe: fragment flanking *Eco*RI. Sample as in B.

Figure 7 shows the cloning strategy used for the construction of pY05. The locations of the primers used for PCR are indicated by arrows. The coding region for HAS within the λ2LK fragment is indicated by the thick line. The restriction enzymes used in the digestion of the DNA are indicated beside the corresponding arrows.

Figure 8 shows the cloning strategy used for the construction of pY011. The two segments of the HAS coding region are indicated as thick dark lines.

Figure 9 shows the cloning strategy used for the construction of pY012 and pY014. The copy of the HAS coding region derived from PCR amplification (thick dark line) present in pY011 was replaced almost completely with the corresponding fragment from λ2LK DNA (lighter thick line).

Figure 10 shows the cloning strategy used for the construction of pY016. The λpLpR promoters and the cI857 repressor gene are indicated as thick black and striped arrows, respectively. The HAS coding region is indicated as a stippled thickened segment.

Figure 11 shows the cloning strategy used for the construction of pY019. The designation of the different fragments is as in Fig. 10, and the added terminators are indicated as fd-term.

Figure 12 shows the controlled expression of HAS in *E. coli*. The molecular weight size standards are indicated on the right hand margin. Lane 1a: pY09, 30°C; lane 1b: pY09, 42°C; lane 2a: pY016, 30°C; lane 2b: pY016, 42°C; lane λ-: λgt11; lane λ+: λ2LK.

Figure 13 shows the growth (OD_{600}) and HA production by recombinant *S. equi* 68222 strains containing plasmid pY09 and the HAS expression vectors pY016 and pY019.

Figure 14 shows the analysis of HPLC fractions for HA synthase activity. The upper panel shows the protein profile of the various fractions; the molecular weight markers are indicated on the left hand side. The lower panel shows the HA synthase activity and the salt concentration of each fraction.

Figure 15 shows the analysis of HPLC fractions for HA synthase activity. The upper panel shows the protein profile of the various fractions; the molecular weight markers are indicated on the left hand side. The lower panel shows the HA synthase activity, the conductivity, and the OD_{280} of each fraction.

Figure 16 shows the molecular weight distribution of HA in fractions of the reaction mixture of Example 11, using HAS to synthesize hyaluronic acid in vitro. The reaction mixture was subjected to gel filtration on Sephacryl S-1000. The left peak of the elution profile represents radiolabelled HA; the right peak represents smaller radiolabelled precursors.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description of the invention is provided to aid those skilled in the art in practicing the same. Even so, the following detailed description should not be construed to unduly limit the present invention, as modifications and variations in the embodiments herein discussed may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited in the present disclosure are herein incorporated by reference in their entirety.

General Recombinant DNA Techniques

The conventional molecular techniques employed were those described by Sambrook (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Cleavage of DNA with restriction enzymes was performed according to the manufacturers' instructions. In general, 1 µg of plasmid DNA was cut with 1 U of enzyme in 20 µl of solution; temperature and incubation times depended on the enzyme used, but were usually 1 hour at 37°C. After incubation, DNA fragments were purified in LMP agarose gels (BRL, U.S.A.) in 40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA, and then

eluted from the agarose with a Geneclean(TM) kit (BIO101 Inc., La Jolla, CA, U.S.A.).

For the filling in reaction at the 5' end, the DNA was treated for 15 minutes at 15°C with 10 U of Klenow polymerase. To dephosphorylate the DNA cut with the restriction enzymes, calf intestine alkaline phosphatase (Boehringer Mannheim, Germany) was used. The reaction was conducted using 1 U of alkaline phosphatase in 20 µl at 37°C for 30 minutes. When necessary, DNA methylation was carried out using the appropriate methylase according to the manufacturer's instructions. For ligation reactions, T4 DNA ligase (Boehringer Mannheim, Germany) was used at a concentration of 1 U per 0.5 µg of DNA in a reaction volume of 20 µl at 14°C for 16 hours.

Oligonucleotides were synthesized on an Applied Biosystems 380 B DNA synthesizer according to the manufacturer's instructions.

The *Streptococcus equisimilis* D181 strain was obtained from the Rockefeller University collection, while the *Streptococcus equi* 68222 and *Streptococcus zooepidemicus* 68270 strains were supplied by the "Istituto Sieroterapico Belfanti" in Milan, and the *Streptococcus sanguis* (Challis) strain was obtained from Dr. Laplace, Jena, Germany. Bacteria were grown in Todd-Hewitt broth or Brain Heart Infusion (BHI) (DIFCO, MI, USA), and plated on culture medium containing 1.5% agar.

Purification of HAS from the protoplastic membranes of the streptococci and production of the antibodies used to isolate the HAS-producing clones has been described by Prehm and Mausolf (Biochem. J., 1986, 235: 887-889).

Genomic DNA was isolated as follows. A colony of streptococci was inoculated into 5 ml of Todd-Hewitt

broth containing 40 mM D,L-threonine and 30 mM glucose, and left to grow while being shaken overnight at 37°C. The culture was then diluted in 100 ml of Todd-Hewitt broth containing threonine alone. To this were then added cysteine (final concentration: 6 mM) at OD_{600} = 0.03 and hyaluronidase (3 units/ml) at OD_{600} = 0.1. Growth was stopped at OD_{600} = 0.5, and the bacteria harvested by centrifugation at 5,000 rpm, washed twice with 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, at 0°C, resuspended in 8.75 ml of said buffer, and incubated with lysozyme (5mg/ml) and hyaluronidase (1.5 units/ml) for one hour at 37°C. Proteinase K and Pronase B were then added (250 µg/ml each) and the solution was left for another 30 minutes at the same temperature. SDS was then added to a final concentration of 0.5%, and incubation was continued at 37°C until the solution became clear, indicating bacterial lysis. NaCl and CTAB (Sigma) were then added to a final concentration of 0.7 M and 1.13%, respectively. The solution thus obtained was extracted once with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). The phases were separated by centrifugation at 6,000 rpm for 10 minutes. The aqueous phases were harvested and the DNA was precipitated with 0.6 volumes of isopropanol.

Nucleotide sequences were determined by Sanger's method (Sanger et al., Proc. Natl. Acad. Sci. USA, 1977, 74: 5463-5467) using a T7 DNA Polymerase sequencing kit (Pharmacia, Sweden).

Plasmid DNA was purified with a kit from Quiagen for plasmidic preparations (DIAGEN GmbH, Duesseldorf, Germany).

Plasmid DNA was sequenced as described by Chen and Seeburg (DNA, 1985, 4: 165-170). DNA amplified by the Polymerase Chain Reaction (PCR) (Saiki et al., Science, 1988, 239: 487-491) was sequenced as described by Both

et al. (Anal. Biochem., 1991, 199: 216-218) using primers from the inner nucleotide sequence of the HAS gene. The DNA of λgt11/2LK-D was sequenced with an fmol(TM) DNA sequencing kit (Promega, Madison, WI, USA) using a linear amplification protocol.

HAS Genes and Proteins

The nucleic acid sequence and polypeptide disclosed herein, or their biologically functional equivalents, can be used in accordance with the present invention. The term "biologically functional equivalents," as used herein, denotes fragments, variants, or mutants of nucleic acid sequences or polypeptides exhibiting the same or similar biological activity as the particular nucleic acid sequence and polypeptide shown in Fig. 3.

For example, the nucleic acid sequence depicted in Fig. 3 can be altered by substitutions, additions or deletions that provide for biologically functionally equivalent molecules possessing HA synthetic activity similar to that of HAS. Due to the degeneracy of the genetic code, other DNA sequences which encode substantially the same amino acid sequence as depicted in Fig. 3 can be used in the practice of the present invention. These include, but are not limited to, nucleotide sequences comprising all or portions of the HAS gene depicted in Fig. 3 which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Similarly, the HAS protein, or derivatives thereof, of the present invention include, but are not limited to, those containing the amino acid sequence substantially as depicted in Fig. 3, including altered sequences in which physiologically functionally equivalent amino acid residues are substituted for residues within the sequence, also resulting in a silent

change. For example, one or more amino acid residues within the sequence can be substituted with another amino acid of similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

While the HAS gene of the present invention preferably encodes the protein shown in Fig. 3, also intended to be encompassed by the present invention are fragments or variants of the HAS gene that encode HAS-like proteins possessing the same or similar HA-synthetic biological activity as that of the naturally occurring HAS gene. These fragments or variants can be truncated forms of the protein wherein amino acids from the N-terminal end, C-terminal end, and/or middle of the protein are deleted, or wherein one or more amino acids have been inserted into the natural sequence. Combinations of the foregoing are also encompassed by the present invention.

Also encompassed by the present invention are DNA fragments derived from streptococcal strains belonging to groups A and C (Lancefield's classification), comprising nucleotide sequences homologous to the sequence shown in Fig. 3 that encode the HAS enzyme in these organisms. Eukaryotic cDNA sequences encoding HAS are also encompassed by the present invention.

It is contemplated that all such fragments, variants, or mutants code for HAS-like proteins that possess substantially the same amino acid sequence as naturally occurring HAS, thereby facilitating the synthesis of HA in a manner similar to that of naturally occurring HAS.

The variants of HAS should possess more than 80% homology, preferably more than 90% homology, and most preferably more than 95% homology, to natural HAS. To determine this homology, two proteins are aligned so as to obtain a maximum match using gaps and inserts. Homology is determined as the product of the number of matched amino acids divided by the number of total amino acids plus gaps and inserts multiplied by 100.

In addition, the recombinant HAS encoding nucleic acid sequence of the present invention may be engineered so as to modify processing or expression of HAS. For example, and not by way of limitation, a signal sequence may be inserted upstream of the HAS encoding sequence to permit secretion of HAS, thereby facilitating harvesting or bioavailability.

Additionally, a given HAS protein can be mutated in vitro or in vivo to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including, but not limited to, in vitro site-directed mutagenesis (Hutchinson et al. (1978) J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

Expression Vectors for HAS

The vectors contemplated for use in the present invention include those into which DNA sequences as discussed herein can be inserted, along with any necessary operational elements. Such vectors can then be subsequently transferred into prokaryotic or eukaryotic host cells and replicated therein. Preferred vectors are those whose restriction sites have been well documented and which contain the operational elements preferred or required for transcription of the DNA sequence.

Certain embodiments of the present invention employ vectors which would contain the DNA sequences described herein. It is preferred that all of these vectors have some or all of the following characteristics: (1) possesses a minimal number of host-organism sequences; (2) be stably maintained and propagated in the desired host; (3) be capable of being present in high copy number in the desired host; (4) possess a regulatable promoter positioned so as to promote transcription of the gene of interest; (5) have at least one marker DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the DNA sequence will be inserted; and (6) contain a DNA sequence capable of terminating transcription.

The cloning vectors capable of expressing the DNA sequences of the present invention contain various operational elements. These "operational elements" can include at least one promoter, at least one Shine-Dalgarno sequence and initiator codon, and at least one termination codon. These "operational elements" may also include one or more of the following: at least one operator, at least one leader sequence for proteins to be exported from intracellular space, at least one gene for a regulator protein, and any other DNA sequences

necessary or preferred for appropriate transcription and subsequent translation of the cloned HAS DNA.

Certain of these operational elements may be present in the preferred vectors of the present invention. It is contemplated that any additional operational elements which may be required may be identified and added to these vectors using methods known to those of ordinary skill in the art, such as those described by Sambrook et al., supra.

Regulators

Regulators serve to prevent expression of the DNA sequence in the presence of certain environmental conditions and, in the presence of other environmental conditions, will allow transcription and subsequent expression of the protein coded for by the HAS DNA sequences. In particular, it is preferred that regulatory segments be inserted into the vector such that expression of the DNA sequence will not occur, or will occur to a greatly reduced extent, in the absence of, for example, isopropylthio-beta-D-galactoside (IPTG). In this situation, the transformed microorganisms containing the DNA sequence may be grown to a desired density prior to initiation of the expression of HAS. Expression of the desired protein is induced by addition of a substance to the microbial environment capable of causing expression of the DNA sequence after the desired cell density has been achieved.

Promoters

The expression vectors must contain promoters which can be used by the host organism for expression of its own proteins. While the lactose promoter system is commonly used, many other well known viral and microbial

promoters have been isolated and characterized, enabling one skilled in the art to use them for expression of HAS and fragments or variants thereof.

Transcription Terminators

The transcription terminators contemplated herein serve to stabilize the vector. In particular, those sequences described by Rosenberg et al. (1979) Ann. Rev. Genet. 13:319-353 are contemplated for use in the present invention.

Non-translated Sequences

It may also be desirable to construct the 3' or 5' end of the coding region to allow incorporation of 3' or 5' non-translated sequences into the gene transcript. Included among these non-translated sequences are those which stabilize mRNA, as disclosed by Schmeissner et al. (1984) J. Mol. Biol. 176:39-53.

Ribosome Binding Sites

The microbial expression of foreign proteins requires operational elements which include ribosome binding sites. A ribosome binding site is a sequence which a ribosome recognizes and binds to in the initiation of protein synthesis as set forth in Gold et al., Ann. Rev. Microbiol. 35:557-580 and Marquis et al. (1986) Gene 42:175-183. A preferred ribosome binding site is GAGGCGCAAAAA(ATG).

Leader Sequences and Translational Couplers

Additionally, it is preferred that DNA coding for an appropriate secretory leader (signal) sequence be present at the 5' end of the DNA sequence, as set forth by Watson, M.E. in Nucleic Acids Res. 12:5145-5163, if the protein is to be excreted from the host cytoplasm.

The DNA for the leader sequence must be in a position that allows the production of a fusion protein in which the leader sequence is immediately adjacent to and covalently joined to HAS, i.e., there must be no transcription or translation signals between the two DNA coding sequences.

In some species of host microorganisms, the presence of an appropriate leader sequence will allow transport of the completed protein into the periplasmic space, as in the case of some *E. coli*. In the case of certain *E. coli*, *Saccharomyces* and strains of *Bacillus* and *Pseudomonas*, the appropriate leader sequence will allow transport of the protein through the cell membrane and into the extracellular medium. In this situation, the protein may be purified from extracellular protein.

An additional DNA sequence can be located immediately preceding the DNA sequence which codes for HAS. The additional DNA sequence is capable of functioning as a translational coupler, i.e., it is a DNA sequence that encodes an RNA which serves to position ribosomes immediately adjacent to the ribosome binding site of the inhibitor RNA with which it is contiguous. The translational coupler may be derived using the DNA sequence TAACGAGGCGCAAAAAATGAAAAAGACAG CTATCGCGATCTTGGAGGATGATTAAATG and methods currently known to those of ordinary skill in the art related to translational couplers.

Translation Terminators

The translation terminators contemplated herein serve to stop the translation of mRNA. They may be either natural, as described by Kohli, J., Mol. Gen. Genet. 182:430-439, or synthetic, as described by Pettersson, R.F. (1983) Gene 24:15-27.

Selectable Markers

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host microorganism. In one embodiment of the present invention, the gene for ampicillin resistance is included in the vector. In other plasmids, the gene for tetracycline resistance or the gene for chloramphenicol resistance can be included.

Such a drug resistance or other selectable marker facilitates the selection of transformants. Additionally, the presence of such a selectable marker in the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. A pure culture of the transformed host microorganisms would be obtained by culturing the microorganisms under conditions which require the induced phenotype for survival.

The operational elements discussed herein are routinely selected by those of ordinary skill in the art in light of prior literature and the teachings contained herein. General examples of these operational elements are set forth in B. Lewin (1983) Genes, Wiley & Sons, New York. Various examples of suitable operational elements may be found in the vectors discussed above, and may be gleaned via review of the publications discussing the basic characteristics of the aforementioned vectors.

Upon synthesis and isolation of all the necessary and desired component parts, the vector can be assembled by methods generally known to those of ordinary skill in the art. Assembly of such vectors is within the ordinary skill in the art, and, as such, is capable of being performed without undue experimentation.

Multiple copies of the DNA sequences of the present invention and their accompanying operational elements may be inserted into each vector. In such case, the host organism would produce greater amounts per vector of HAS. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and transcribed in an appropriate host cell.

Other Host Microorganisms and Cells

Vectors suitable for use in microorganisms other than *E. coli* are also contemplated for use in the present invention. Such microorganisms include, for example, *Streptococci*, *Bacillus*, *Pseudomonas*, *Salmonella*, *Serratia*, and yeast.

Also contemplated within the scope of the present invention is the expression of HAS in vertebrate or invertebrate cells, including, for example, Chinese Hamster Ovary cells, VERO cells, HeLa cells, WI38 cells, BHK cells, COS cells, and MDCK cells, as well as insect cells, such as those of *Spodoptera frugiperda*.

For expression in *Bacillus*, preferred vectors should include a regulated promoter such as the alpha amylase promoter, the subtilisin promoter, the P-43 promoter, or the spac-126 promoter. Useful transcription terminators include rrn and rrn BT.T. Transcriptional start sites and leader peptides can be chosen from among those from *B. amyloliquefaciens* neutral protease, *B. amyloliquefaciens* alpha-amylase, and *B. subtilis* subtilisin. Useful antibiotic markers are Kan^r and Cam^r. Ribosome binding sites can be obtained from the *B. amyloliquefaciens* neutral protease and *B. amyloliquefaciens* alpha-amylase genes. A preferred expression system in hosts of the genus *Bacillus*

involves the use of plasmid pUB110 as the cloning vehicle.

For expression in *Pseudomonas*, promoters can be selected from Trp, Lac, and Tac. Useful transcriptional start sites and leader peptides can be obtained from the phospholipase C and exotoxin A genes. Useful antibiotic markers are those for sulfonamides and streptomycins. A useful ribosome binding site can be obtained from the Trp promoter of *E. coli*. Particularly preferred vectors would employ the plasmid RSF1010, and derivatives thereof.

In the case of yeast, useful promoters include Gal 1 and 10, Adh 1 and 11, and Pho 5. Transcription terminators can be chosen from among Cyc, Una, Alpha Factor, and Sac 2. Transcriptional start sites and leader peptides can be obtained from the invertase, acid phosphatase, and Alpha factor genes. Useful selection markers are Ura 3, Leu 2, His 3, and Tap 1.

Finally, in the case of expression in mammalian cells, the DNA encoding HAS should have a sequence efficient at binding ribosomes. Such a sequence is described by Kozak in Nucleic Acids Research (1987) 15:8125-8132. The HAS-encoding fragment can be inserted into an expression vector containing a transcriptional promoter and a transcriptional enhancer as described by Guarente in Cell (1988) 52:303-305 and Kadonaga et al. (1987) Cell 51:1079-1090. A regulatable promoter as in the Pharmacia plasmid pMSG can be used, if necessary or desired. The vector should also possess a complete polyadenylation signal as described by Ausubel et al. (1987) in Current Protocols in Molecular Biology, Wiley, so that mRNA transcribed from the vector is properly processed. Finally, the vector may also contain the replication origin and at least one antibiotic resistance marker from a plasmid such as pBR322, to

allow replication and selection in *E. coli*.

In order to select a stable cell line that produces HAS as described herein, the expression vector can carry the gene for a selectable marker such as a drug resistance marker or a complementary gene for a deficient cell line, such as a dihydrofolate reductase (dhfr) gene for transforming a dhfr cell line, as described by Ausubel et al., supra. Alternatively, a separate plasmid carrying the selectable marker can be cotransformed along with the expression vector.

Vectors for mammalian cells can be introduced therein by several techniques, including calcium phosphate:DNA coprecipitation, electroporation, or protoplast fusion. Coprecipitation with calcium phosphate as described by Ausubel et al., supra, is the preferred method.

Preferred vectors include, for example, a PSVT7 eukaryotic expression plasmid.

EXAMPLE 1

Isolation of a λgt11 clone expressing HAS

A genomic library was constructed using DNA of *Streptococcus equisimilis* D181. The genomic DNA was partially digested with the restriction enzyme *Alu*I, and the fragments ranging in size from 2 to 4.5 kb were isolated by sucrose gradient centrifugation (J.H. Weis in Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., 1989). The isolated fragments were dephosphorylated, methylated with *Eco*RI methylase, and bound to *Eco*RI linkers (Wu et al., 1987, in Guide to Molecular Cloning Techniques, S. Berger et al., eds., Academic Press, San Diego). The linker-containing DNA was digested with *Eco*RI, separated from the other fragments, ligated to λgt11 DNA, and packaged in infectious virions using the "one-strain-packaging"

system SMR10 described by Rosemberg et al. (Gene, 1985, 38: 165-175) The packaged DNA was used to transfect *E.coli* Y1089 (Stratagene). The library was screened using polyclonal antibodies directed against HAS, previously affinity purified on the protein itself immobilized on CNBr-Sepharose. Cross-reactive antibodies to *E.coli*/λgt11 lysates were removed as described by Sambrook et al. Positive clones were identified on plates without using the lacZ inducer, IPTG, as described by Huynh et al. (1986, in DNA Cloning: A Practical Approach, D.M Glover, ed., Vol. 1, IRL Press, Oxford, England), indicating that the recombinant clones contained a promoter functioning in *E.coli*. Ten clones were isolated from 50,000 lysis plaques. These clones were purified and amplified as described in Sambrook et al. Proteins were extracted with chloroform/methanol.

Following electrophoresis on a polyacrylamide gels in the presence of SDS (SDS-PAGE) and Western blotting, clones expressing the entire HAS protein were identified and isolated. One of these clones expressed two proteins, of about 52 and 56 kDa, which were recognized by the antibodies (Fig. 1, B). A similar protein pattern was observed in protoplastic membranes extracted from the streptococci (Fig. 1, A). The proteins of the λt11 lysate (Fig. 1, C) were not recognized by the antibodies. This clone, designated λgt11/2LK, containing a DNA insert of about 3.1 kb, was selected, and when digested with *Eco*RI, generated fragments of 2.1, 0.33 and 0.67 kb (Fig. 2). The 2.1 kb fragment expressing HAS was subcloned in λgt11 to generate λgt11/2LK-D, which was used for subsequent analysis. A deposit thereof has been made at the DSM.

EXAMPLE 2

Amino acid sequence analysis

HAS was purified from both *S. equisimilis* D181 and λgt11/2LK lysates as described by Prehm and Mausolf (Biochem. J., 235: 887-889, 1986). Purified HAS (200 µg) was dissolved in 200 µl of 6 M urea, diluted with 400 µl of 20 mM Tris-HCl, pH 7.8, and digested with 5% trypsin or V8-protease for 24 hours. The mixture was acidified with trifluoroacetic acid and the peptides centrifuged at 10,000 rpm for 3 minutes. The supernatant was applied to a C18 reverse-phase HPLC column (VYDAC 218 TP54). Peptides were eluted in 30 minutes with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 70% acetonitrile with a flow rate of 1 ml/min. Elution was monitored at 220 nm. The fractions were dried and analyzed in a gas phase sequencer. The proteolytic fragments of the synthase, both recombinant (λgt11/2LK) and from streptococci, were isolated and analyzed. The same sequence was found in the two preparations, indicating that the recombinant protein was indeed HAS.

EXAMPLE 3

Nucleotide sequence analysis

The double-stranded DNA was isolated from λgt11/2LK-D by Manfiolette's method (Manfiolette et al., Nuc. Acids Res., 1988, 16: 2873-2884). It was not possible to subclone the whole 2.1 kb fragment in plasmids such as pGEM-3Z or 7Z (Promega, Madison, WI, USA), pUC-18 (New England BioLabs, Beverly, MA, USA) or pBluescript SK+ (Stratagene, La Jolla, CA, USA), which would have allowed the determination of the nucleotide sequence by a conventional strategy. The insert cloned in *E. coli* was always shorter than the original. It was, however, possible to subclone the right-hand fragment of

about 1.27 kb from the *Xba*I site to the *Eco*RI site (see Fig. 2) in pGEMR-7Z, indicating that the region on the left of about 0.86 kb was responsible for the plasmid instability. The *Xba*I-*Eco*RI fragment was subcloned into smaller fragments and the sequence was determined by plasmid sequencing. The left hand portion of λgt11/2LK-D was sequenced by using a number of different approaches. The extreme left hand part was sequenced on the λgt11/2LK-D DNA itself using an fmol DNA sequencing kit (Promega), primer 2 (5'-TTGACACCAGACCAACTGGTAATG-3') labelled with ^{32}P, and linear amplification. Briefly, 17 µl of reaction mixture containing 150 ng of λgt11/2LK-D DNA, 50 mM Tris-HCl, pH 9.0, and 2 mM $MgCl_2$ were mixed with a suitable mixture of d/ddNTP, covered with mineral oil, and exposed to 30 cycles of Polymerase Chain Reaction (PCR) (30 seconds at 95°C, 30 seconds at 65°C) after initial denaturation at 95°C for 2 minutes. Further sequence information was obtained from the λgt11/2LK insert of about 3.1 kb which had been amplified by PCR, using primers flanking the *Eco*RI cloning site (primer 1: 5'-GGTGGCACGACTCCTGGAGCCCG-3'; primer 2). Initial denaturation at 95°C for 2 minutes was followed by 35 cycles at 95°C for 1 minute, 45°C for 1 minute, and 72°C for 5 minutes. The product of this amplification was sequenced using primers from the inner sequence of HAS (primer 3: 5'-AGGAATGTCACAACCT-3'; primer 4: 5'-CCCCTAGAGAGTCTAGA-3'). Thirdly, λgt11/2LK-D DNA was sequenced directly according to Snyder's modified protocol (Snyder et al., Meth. Enzymol., 1987, 154: 107-128). Five µg of DNA were digested with *Kpn*I and *Sac*I, extracted with phenol, precipitated with ethanol, and resuspended in 20 µl of water. The DNA was denatured with 2 µl of 2 M NaOH, 2 mM EDTA for 10 minutes at 37°C. It was then precipitated with 13 µl of 1.5 M Na acetate and 2.5 volumes of ice

cold ethanol (-20°C), and left in a bath of methanol and dry ice for 10 minutes. After centrifugation, the DNA was resuspended in 15 µl of 7.5 mM Tris-HCl, 7.5 mM dithiothreitol, 5 mM $MgCl_2$, pH 7.5, 0.7 ng of primers 1 or 2, labelled with ^{32}P, and incubated at 55°C for 15 minutes. Thereafter, the protocol described for the plasmidic DNA sequence was followed. Finally, restriction fragments of λgt11/2LK generated by digestion with the enzymes *NdeI*, *NheI* and *RsaI* were cloned in plasmids and sequenced according to the protocol described. The sequenced fragments are shown in Fig. 2. The amino acid sequence derived from the nucleotide sequence was compared to that of the HAS peptides, and the two proved to be identical.

Figure 3 shows the complete streptococcal HAS nucleotide sequence and that of the flanking regions with their amino acid sequences. The sequences obtained from the isolated peptides are underlined. Starting from the ATG codon corresponding to nucleotides 349-351, there is an open reading frame of 1566 bp, terminating with a TAG codon at nucleotides 1934-1936. This fragment of DNA can encode a protein of 522 amino acids with an Mr of 56,624. Despite the presence of an ATG codon in position 424-426 which could itself correspond to an initiation codon, the 349-351 codon is probably correct. Indeed, it possesses a Shine-Delgarno sequence (GGAAGG) 4 bp before the initiation ATG, very similar to the sequence linked to the ribosomes seen in other Gram positive bacteria. A possible promoter for Gram positive bacteria has been identified, shown as an unbroken line above the sequence in Fig. 3, at -35 and -10 (TATACA and TAAAAT), with an ideal space of 16 bp. The region immediately before the start is rich in AT (74% vs 57% in the encoding region) in a manner that is characteristic of a promoter region of Gram positive

bacteria. Within this AT-rich region there are two inverted sequences (Fig. 4) which could be functional in regulating gene expression. At the end of the complete encoding sequence are two additional inverted sequences (Fig. 4) which could form a potential loop of 1653 bp. Three palindromic sequences are also shown, two inside the encoding region and one after the terminal TAG codon (shown in Fig. 2 as a broken line above the sequence), the function of which is as yet unknown. Streptococcal HAS has been found in protoplastic membranes. It is therefore to be expected that the mature protein is preceded by a signal peptide.

EXAMPLE 4

Hydrophobic profile of HAS

Figure 5 shows the hydrophobic profile of HAS. Starting from the initiation ATG, the first 33 amino acids have the characteristics of a typical bacterial "leader" sequence (Vlasuk et al., J. Biol. Chem., 1983, 258: 7141-7148): a portion of 12 basic amino acids followed by a hydrophobic core of 14 amino acids terminating with 7 hydrophilic amino acids. There is a putative cleavage site at amino acid 35 for the removal of the signal peptide, whose 3.5 kDa could reduce the molecular weight of the processed protein from 56 kDa to 53.1 kDa. This is the weight determined in previous studies (Prehm and Mausolf, Biochem. J., 1986, 235: 887-889) and, indeed, different quantities of the processed and wild forms have been found in protoplastic membranes of streptococci and in λgt11/2LK lysates in *E. coli* (Fig. 1). Therefore, the cleavage of the signal peptide from HAS explains the two forms of the protein observed in the Western blot in Fig. 1.

EXAMPLE 5

Southern blotting analysis in other streptococci

Southern blotting experiments were carried out to determine whether the gene encoding HAS is conserved in other HA-producing streptococci. Five μg of genomic DNA digested with various restriction enzymes (Fig. 6) were subjected to electrophoresis in a 1% agarose gel. The DNA was then depurinated (0.25 N HCl for 10 minutes, twice), denatured (1.5 M NaCl, 0.5 M NaOH for 25 minutes, twice), neutralized (1 M Tris-HCl, pH 6.5, 2 M NaCl for 30 minutes, twice), and lastly transferred overnight from the gel to a nylon Hybond-N membrane (Amersham, England) by the Southern blotting technique.

The DNA was then fixed to the membrane by exposure to UV irradiation for 3 minutes. A "DIG Non-radioactive DNA Labeling and Detection Kit" (Boehringer Mannheim, Germany) was used to label the probe and in subsequent stages of the experiment, following the manufacturer's instructions. The various purified fragments (about 400 ng each) used as probes were labelled in a non-radioactive fashion by random priming at 37°C for 1 hour. The filters onto which the DNA had been transferred were prehybridized in at least 20 ml of hybridization solution (5xSSC, 1% w/v blocking reagent, 0.1% w/v N-laurosylsarcosine sodium salt, 50% formamide) for 2 hours at 42°C, and then hybridized overnight at the same temperature following addition of the labelled, denatured probe to the solution. The filters were then washed and developed. The results are shown in Fig. 5.

The DNA of all three HA-producing strains (*S. equi*, *S. zooepidemicus*, and *S. equisimilis*) hybridized with the probe containing the entire insert of 3.1 kb of λgt11/2LK (Fig. 6A), while the result was negative with the *S. sanguis* strain which does not in fact produce HA. Surprisingly, numerous bands were observed in lanes

containing the *S. equisimilis* DNA, indicating the presence of numerous copies of the DNA sequence used as a molecular probe. Two other probes were used to determine whether this phenomenon was correlated with the HAS gene or with DNA regions flanking it: these were the flanking *Eco*RI fragment of 0.67 kb and the *Xba*I-*Pst*I fragment inside the HAS coding region. The results are shown in Figs. 6B and 6C. The unexpected pattern was observed only with the first probe and only in *S. equisimilis* D181. In the other HA-producing strains tested, the probe containing the entire gene showed similar behavior and hybridized with a single fragment.

These results indicate that the HA synthase gene, or genes homologous thereto, are conserved in all the streptococcal strains analyzed, thus demonstrating the importance thereof in HA synthesis. The HAS genes of streptococcal strains can be isolated by employing the 3.1 kb insert of λgt11/2LK, or fragments thereof, as probes by techniques well known in the art. The repetitive profile observed in D181 suggests that the HAS gene is flanked by a DNA segment peculiar to this organism and of which 5-10 copies are to be found within the chromosome. This could be an insertion sequence or a transposon.

EXAMPLE 6

Construction of an expression vector for HAS synthesis

The identification of promoters active in Lancefield's group C streptococci has been described in Italian patent application No. PD92A000109 filed on 19 June 1992. One of the promoter fragments found to be active in this study which contains the λpL and pR promoters in tandem was used to construct an expression vector for HAS as outlined below. Obviously, any expression vectors containing promoter sequences active

in streptococci, *E. coli*, other bacterial species, or eukaryotic cells can be used in the construction of plasmids for the overexpression of HAS.

The HAS encoding fragment could not be cloned directly from λgt11/2LK-D due to the presence of unsuitable internal restriction sites. Therefore, attempts were made to introduce suitable restriction sites at both ends of the fragment by PCR amplification of the insert with oligonucleotides containing *Bam*HI sites (primer F11: 5'-TTGGATCCGCGACGACTCCTGGAGCCCG-3' and primer R12: 5'-TTGGATCCCACCAGACCAACTGGTAATG-3'). The fragment was amplified successfully using λgt11/2LK-D as template and the following amplification protocol: 1 cycle of 2 min 94°C, 2 min 45°C, 5 min 72°C followed by 34 cycles of 1 min 94°C, 2 min 34°C, 5 min 72°C. Unfortunately, all attempts to clone the entire amplified fragment in plasmid vectors failed, suggesting a possible toxic effect of the protein in *E. coli* or the presence of streptococcal sequences that could cause plasmid instability. The amplified fragment was digested with the restriction enzyme *Xba*I, and the right-hand part of the fragment (from the unique internal *Xba*I site to the end) was cloned, yielding the plasmid pY05. The cloning strategy is outlined in Fig. 7. In order to clone the left-hand segment, the primers ST2 and ST3 were used. These primers have the following sequences: ST2: 5'-CTCTCGAGTTAGGAGGAAGGTCATATGACAGTA-3'; ST3: 5'-CCTAGAGTCTAGATCAGGATTTT-3'. ST2 is located at the beginning of the HAS coding sequence and includes the ribosome binding site and an artificially introduced *Xho*I site to facilitate subsequent cloning. ST3 is located near the internal *Xba*I site of the cloned insert. These primers were used to amplify a DNA fragment from λgt11/2LK using the following protocol: 35 cycles of 1 min 95°C, 1 min 45°C, 1 min 70°C. The

amplified fragment was subsequently purified and digested with the restriction enzymes *Xba*I and *Xho*I. The purified fragment was digested with *Xho*I-*Xba*I, re-purified, and ligated with the purified *Xba*I-*Bam*HI fragment from pYO5 and *Xho*I-*Bam*HI digested pGEM-7Z, and transformed into *E. coli* HB101 (Promega) to yield plasmid pY011 (Fig. 8). Since the insert of pY011 was derived entirely from PCR amplification and, therefore, might contain some mutations, as much of the insert as possible was replaced with DNA derived directly from genomic cloning. The *Nhe*I-*Eco*RI fragment of λgt11/2LK, which contains most of the HAS coding sequence, was subcloned into plasmid pGEM4 to yield pY012 (Fig. 9). The *Hin*dII fragment of pY011 was subsequently replaced by the corresponding fragment of pY012 as outlined in Fig. 9 to yield plasmid pY014. The HAS encoding sequence was placed under the transcriptional control of the λpLpR promoters, as outlined in Fig. 10, by carrying out a triple ligation containing the *Xho*I-*Eco*RI fragment of pY014, an *Xho*I-*Bam*HI fragment from plasmid pGEM-λprom*Bam*HI containing the λpLpR promoters and the cI857 repressor gene, and *Bam*HI-*Eco*RI digested pCK17, a cloning vector derived from a cryptic lactococcal plasmid (Anderson et al., 1985, FEMS Microbiol. Lett., 30: 193-196). The resulting HAS expression vector was designated pY016.

Plasmid pY016 does not contain any transcription terminators downstream of the HAS gene. Since it is possible that the presence of terminators may have a positive effect on cloned gene expression by giving rise to more stable mRNA, transcription terminators were added to pY016 in the following manner. The *Eco*RI-*Nar*I fragment of plasmid pJLA501 (Medac) containing the *E. coli* fd terminators, was ligated to *Eco*RI-*Cla*I digested pY016, and the ligation mix was transformed into *E. coli*

HB101 to yield pY019 as outlined in Fig. 11.

EXAMPLE 7

Controlled expression of HAS in *E. coli*

Plasmids pY016 and pY09 (the expression vector not containing the HAS encoding gene) were introduced into *E. coli* ED8739 (Murray et al., Mol. Gen. Genet., 150: 53-61, 1977) by transformation, and transformants were selected on LB agar medium containing 20 μg/ml of chloramphenicol (Cm). It is well known that the activity of the λpLpR promoters is blocked by the temperature sensitive repressor cI857 at 30°C, but that the repressor is inactivated at 37°C or above, thus allowing expression of genes under control of the promoters.

To determine if expression of the HAS gene was thermoinducible, *E. coli* ED8739 containing pY016 or pY09 was grown in LB containing Cm (20 μg/ml) at 30°C overnight and subcultured into 2 separate LB cultures which were subsequently incubated at 30 and 42°C for 3h. An aliquot of 1 ml of each culture was transferred to a 1.5 ml Eppendorf tube, and the cells were pelleted by centrifugation at 15,000xg for 30 sec. Total proteins were extracted by resuspending the pellet in 0.5ml of extraction buffer (2% SDS, 5% β-mercaptoethanol) and heating to 95°C for 3 min. The samples were centrifuged for 3 min at 15,800xg, aliquots of 40 μl of each supernatant were loaded on a 10% polyacrylamide gel, and proteins were separated by electrophoresis at 18mA for 16h. The proteins were transferred to nitrocellulose by Western blotting. Detection of HAS was carried out using polyclonal rabbit antibodies raised against a synthetic peptide (sequence: RADAKGKLQPDLAEKVDVSE) corresponding to amino acids 76-95 of the HAS sequence (see Fig. 3). The results are shown in Fig. 12.

E. coli ED8739 containing the expression vector pY016 grown at 42°C (lane 2b) gives rise to two protein bands of the same size as those observed in the original λgt11/2LK clone (lane λ+). Estimation of the total amount of protein produced indicates that HAS corresponds to about 10% of total cellular protein. The presence of the two forms of HA synthase indicates that the signal sequence is also cleaved efficiently from the protein produced by pY016.

EXAMPLE 8

Production of HA by recombinant strains of *S. equi* 68222 containing HAS expression vectors

To determine if the presence of HAS expression vectors in *S. equi* 68222 results in an increase in HA production with respect to the wild-type strain, *S. equi* 68222 containing the plasmids pY09, pY016 or pY019 were compared with respect to the quantity of HA produced. Plasmid pY09 was the negative control as it contains only the λpLpR promoters and the repressor gene, but not the HAS encoding gene. Plasmids pY09, pY016, and pY019 were transformed into *S. equi* 68222 by electroporation. The basic procedure was as follows: Overnight cultures grown in BHI medium at 37°C in a shaking incubator were diluted 10-fold in fresh medium containing 6,000 units of hyaluronidase per liter and grown under the same conditions until they reached an OD_{600} of approximately 0.5, which corresponds to the mid-exponential phase of growth. The culture was chilled on ice for 10 minutes and the cells were subsequently pelleted by centrifugation at 5,000 rpm for 10 min. The cells were washed twice with an equal volume of ice-cold distilled water and subsequently resuspended in ice-cold distilled water to obtain a final OD_{600} of about 50. An aliquot of 50 µl of this cell suspension was mixed in an Eppendorf

tube with 1-2 µl of plasmid DNA solution at a final concentration of 100 µg/ml. The mixture was transferred to an electroporation cuvette with a spacing of 0.2 mm, inserted in a Genepulser, and subjected to an electric pulse under the following conditions: voltage, 12.5 kv/cm, resistance 200 ohms, capacitance 25 µF. The samples were immediately diluted in 1 ml of fresh BHI medium and incubated for 1-2 h at 37°C to allow plasmid gene expression. To select transformed cells, aliquots of the transformation mixture were plated on BHI plates containing 5 µg/ml Cm. The plates were incubated at 37°C, and transformants appeared within 24-48 h.

Mucoid colonies of *S. equi* 68222 containing pY09, pY016 or pY019 were inoculated from BHI agar plates containing 5 µg/ml Cm into a small volume of BHI plus Cm in Erlenmeyer flasks and grown overnight on a rotating incubator at 37°C. These overnight cultures were diluted 10-fold in fresh medium containing, in addition, glucose at a final concentration of 2% and the acid-base indicator phenol red at a final concentration of 5 mg/l. The phenol red turns yellow when the culture broth becomes acidic due to the production of lactic acid by the streptococci. Since acidic conditions inhibit bacterial growth and HA production, once a yellow colour is observed in the culture medium, a sufficient quantity of 3 N NaOH must be added to raise the pH to neutrality. The cultures were incubated at 37°C in a rotating incubator and samples were taken at regular intervals to follow growth and HA production. Growth was followed by measuring the OD_{600} of the samples. The HA was precipitated from the culture broth by the addition of two volumes of ethanol, and the precipitate was resuspended in an equal volume of distilled water. The HA concentration was measured by the method of Blumenkrantz and Absoe-Hansen (Anal. Biochem., 54:

484-489, 1973).

The results are shown in Fig. 13 and Table 1. The strains containing pY016 and pY019 produced higher levels of HA compared to the strain containing pY09, indicating that the presence of multiple copies of the HAS encoding gene favours the more efficient production of HA. When the quantity of polymer is calculated with respect to the culture OD, it becomes evident that the pY016 and pY019 strains had accumulated approximately 1.4-1.5 times more HA than the pY09-containing strain at the end of the cultivation period.

TABLE 1

Time of culture	OD (600 nm)	HA (mg/L)	YIELD HA mg/L / OD 600nm
T = 3 h 30'			
pYO9	1.107	173.4	170.5
pYO16	0.99	240.5	243
pYO19	1.23	210	210
T = 5 h			
pYO9	2.13	318	149
pYO16	1.8	377	209
pYO19	1.873	358	191
T = 6 h 30'			
pYO9	3.252	418	128.5
pYO16	2.925	462	158
pYO19	2.889	500	173
T = 8 h			
pYO9	4.719	672	142.4
pYO16	4.284	925.6	216
pYO19	4.476	870	194
T = 9 h 30'			
pYO9	5.218	791.4	151.6
pYO16	4.623	1120	242
pYO19	4.606	1137	247
T = 10 h 15'			
pYO9	5.362	836	156
pYO16	4.88	1072	220
pYO19	4.638	1083	233
T = 11 h			
pYO9	5.366	818	152
pYO16	4.446	1095	246
pYO19	4.714	1029	218

It will be obvious to those proficient in the art that the culture conditions can be varied significantly with respect to growth medium and culture conditions while still obtaining a positive effect on the over-expression of the HAS gene.

EXAMPLE 9

Industrial applications of the HAS gene

As demonstrated by the results of Examples 7 and 8, recombinant prokaryotic cells harboring an expressible DNA sequence encoding HAS can be produced which, when grown under appropriate conditions, produce elevated levels of HAS or hyaluronic acid.

In a similar manner, employing techniques well known in the art as described above, vectors for the expression of HAS in eukaryotic cells, such as mammalian cells and insect cells, can also be constructed.

Such recombinant prokaryotic or eukaryotic cells harboring an expressible DNA sequence encoding HAS can be employed as industrial sources of HAS and/or hyaluronic acid. By culturing these cells under appropriate conditions, it will be possible to recover HAS, hyaluronic acid, or both from the cells themselves, from the culture medium, or from both.

EXAMPLE 10

Purification of HAS

In order to achieve in vitro synthesis of HA and exploit the advantages described above, it was necessary to develop a fast and simple method for the purification of active HAS.

HAS can be purified according to the following protocol:

1. *Streptococcus equisimilis* D181, or any alternative cells capable of producing HA such as

related Lancefield's group A or C streptococcal species or recombinant organisms expressing HAS, is grown in the desired volume of Todd-Hewitt broth or any other suitable nutrient medium at a temperature between 30 and 40°C, but preferably at 37°C, to a final OD_{600} greater than 0.1, and preferably about 0.5. Approximately 1 h before cell harvesting, 6,000 units per liter of hyaluronidase can be added to degrade any HA produced.

2. The bacteria are harvested by centrifugation, washed at least two times with a suitable buffer such as cold phosphate buffered saline (PBS), and resuspended in a final volume of between 100 and 1,000 ml, but preferably in 200 ml, of PBS containing approximately 1 mM dithiothreitol.

3. This suspension is subjected to procedures designed to cause cell disruption, such as sonication at 0°C, under conditions sufficient to ensure complete cell disruption while minimizing enzyme inactivation. A wide range of sonication conditions can be used, but those preferred are 15 min. at 120 watts. Subsequent operations are carried out at 4°C.

4. Bacterial debris is removed by centrifugation, and the supernatant is subjected to ultracentrifugation under conditions capable of sedimenting the bacterial membranes, preferably at 40,000 rpm for 30 min.

5. The sediment, which contains the streptococcal membranes, is suspended in a small volume of 50 mM Tris-malonate buffer, or any other suitable buffer, containing approximately 1 mM dithiothreitol, by sonication under mild conditions, for example 30 sec. at 20 watts. The protein concentration is determined, and the suspension is diluted in the same buffer to a final protein concentration of between 1 and 10 mg/ml, but preferably at 3 mg/ml.

6. To extract HAS from the bacterial membranes, a 10% solution of a mild detergent such as digitonin is added to the sample to give a final concentration of between 0.5 and 2%, but preferably 1%. The suspension is stirred in order to ensure thorough mixing for approximately 1 hour, and subjected to ultracentrifugation in order to separate membrane fragments at 40,000xg for 30 min. or other similar conditions.

7. To achieve separation of the synthase from the detergent phase, a number of strategies can be used, but that preferred is the procedure previously described by Parish et al. (Anal. Biochem., 1986, 156:495-502) where phase separation is achieved in certain detergents by the addition of polyethylene glycol (PEG) 6000. To ensure that the synthase separates into the aqueous phase, the protein can be "loaded" with newly synthesized HA to increase its affinity for the aqueous phase.

Therefore, to 5 ml of the supernatant from step 6 the following compounds are added: 1 ml of a 50% solution of PEG 6000, between 0.025 and 0.1 ml, but preferably 0.05 ml, of a 1 M solution of $MgCl_2$, between 0.5 and 5 mg, but preferably 2 mg each, of UDP-N-acetylglucosamine and UDP-glucuronic acid. The mixture is incubated under conditions suitable for the synthesis of HA, for example at 37°C for 30 min., and is subsequently rapidly cooled to 0°C in an ice/salt bath. An additional aliquot of 1 ml of 50% PEG 6000 is added, and the solution is vortexed. The solution becomes turbid, indicating that phase separation has taken place. The suspension is again subjected to ultracentrifugation, for example at 40,000 rpm, for 30 min.

8. In order to separate active HAS from the above mixture, a large number of approaches can be used, including HPLC chromatography. Preferably, the supernatant is subjected to HPLC on an ion-exchange column such as a Waters DEAE-protein Pak 5PW, 7.5 mm x 7.5 mm, which has been equilibrated with a suitable buffer such as 50 mM Tris-malonate buffer, pH 7.0, containing a detergent such as digitonin at a final concentration of about 0.5%. The sample is pumped onto the column at the desired flow rate, preferably at the rate of 1 ml/min. Following loading of the supernatant, the column is washed with the starting buffer, and proteins can be eluted by progressively increasing the salt concentration of the buffer, for example from 0 to 0.5 M, in 30 min. with a constant flow rate of 1ml/min. Fractions of the desired volume can be collected; a volume of 1 ml is suitable.

9. To identify the fractions containing active HAS, a number of determinations are carried out on each fraction. These include: absorbance at 280 nm, conductivity, and HAS activity as determined using radiolabelled precursor as described previously by Prehm (Biochem. J., 1983, 211:181-189). In order to associate the HAS activity with a certain protein, the protein pattern of each fraction can be analyzed by polyacrylamide gel electrophoresis following precipitation of the proteins from a fixed volume of each fraction, for example 200 µl, by standard techniques such as that described by Wessel and Flugge (Anal. Biochem., 1984, 138:141-143).

The results of two independent experiments are shown in Figs. 14 and 15. In both cases, the peak of HAS activity resides in fractions containing an essentially pure protein of approximately 56 kDa, which corresponds to the HAS described above.

These results prove that the 56 kDa protein constitutes the active synthase, and is sufficient to facilitate the synthesis in vitro of HA having characteristics superior to the HA presently isolated from animal or bacterial sources.

EXAMPLE 11

In vitro production of hyaluronic acid of varying molecular weight using purified HAS

Hyaluronic acid of varying molecular weight for different applications can be synthesized in vitro using purified HAS.

A typical protocol is as follows:

1. In a reaction vessel of desired volume, purified HAS is diluted in 50 mM Tris-malonate buffer, or any other suitable buffer, to a final protein concentration of between 0.01 and 1.0 mg/ml, but preferably at 0.1 mg/ml. To the solution, dithiothreitol to a final concentration of approximately 1 mM, $MgCl_2$ to final concentration of approximately 10 mM, UDP-GlcA and UDP-NAcGlc both in concentrations to between 0.1 and 5 mM, but preferably to 1 mM, are added.

2. The mixture is incubated under conditions suitable for the synthesis of HA, for example at 37°C for 4 hours, and subjected to gel filtration on a Sephacryl S-1000 (83 cm x 1 cm) column or any other suitable column. The HA is eluted with PBS or any other suitable buffer.

3. To identify the molecular weight of the HA produced according to steps 1 and 2, radiolabelled precursors are used as described previously by Prehm (Biochem. J., 1983, 181-189). The same conditions for incubation of the mixture and gel filtration are used as described in step 2. Fractions of the eluted material are collected in volumes between 0.1 ml and 2 ml, but

preferably 0.58 ml, and aliquots, preferably 0.2 ml, are taken for determination of radioactivity.

The results are shown in Fig. 16. The molecular weight of the HA synthesized in vitro for 4 hours at 37°C using purified HAS is about 350,000 Da.

The molecular weight of HA synthesized in vitro employing HAS can be varied by varying the incubation period and/or reaction conditions, with the molecular weight varying with varying incubation time and reaction conditions in the presence of HAS. Those reaction conditions which can be varied to influence the molecular weight of the final HA product include, but are not limited to, pH, temperature, etc. The pH can be varied between 4.2 and 8.9; a decrease in pH results in a decrease in molecular weight of HA. With respect to the effect of temperature, this may be varied between 10 to 40°C; a decrease in temperature results in a decrease in molecular weight of the HA produced.

HA produced in this manner possesses the highly desirable property for pharmaceutical and other related applications that it is extremely pure as compared to HA purified from conventional sources, and does not contain any contaminating proteins, pyrogenic or inflammatory substances, or viruses.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

CLAIMS

1. An isolated DNA fragment, comprising a nucleotide sequence encoding hyaluronic acid synthase.

2. The DNA fragment of claim 1, wherein said nucleotide sequence encoding hyaluronic acid synthase is derived from a member of the genus *Streptococcus*.

3. The DNA fragment of claim 2, wherein said member of the genus *Streptococcus* is *Streptococcus equisimilis* D181.

4. The DNA fragment of claim 1, wherein said nucleotide sequence encodes a protein having the amino acid sequence shown in Figure 3.

5. The DNA fragment of claim 1, wherein said nucleotide sequence is the nucleotide sequence shown in Figure 3 from position 349 to position 1917.

6. The DNA fragment of claim 1, wherein said nucleotide sequence comprises a sequence encoding a polypeptide having biological activity functionally equivalent to the amino acid sequence shown in Figure 3.

7. The DNA fragment of claim 6, wherein said DNA fragment is a eukaryotic cDNA.

8. The DNA fragment of claim 6, wherein said nucleotide sequence comprises a variant of the nucleotide sequence shown in Figure 3 in accordance with the degeneracy of the genetic code.

9. The DNA fragment of claim 6, wherein said nucleotide sequence comprises a variant, mutant, or fragment of the nucleotide sequence shown in Figure 3 encoding a polypeptide containing at least one amino acid substitution, addition, or deletion, or a combination of any two or more thereof.

10. The DNA fragment of claim 2, wherein said member of the genus *Streptococcus* belongs to group A or group C.

11. A recombinant expression vector, comprising a nucleotide sequence encoding hyaluronic acid synthase, wherein said vector is capable of expressing said hyaluronic acid synthase in a transformed prokaryotic or eukaryotic cell.

12. A recombinant expression vector, comprising a nucleotide sequence encoding hyaluronic acid synthase, wherein said vector is capable of expressing said hyaluronic acid synthase in a transformed prokaryotic or eukaryotic cell, and wherein said nucleotide sequence is the nucleotide sequence as defined in any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

13. The recombinant expression vector of claim 11, wherein said nucleotide sequence is the nucleotide sequence shown in Figure 3.

14. The recombinant expression vector of claim 13, wherein said vector is a member selected from the group consisting of pY016 and pY019, as shown in Figures 10 and 11, respectively.

15. A prokaryotic or eukaryotic cell transformed with said recombinant expression vector of claim 11.

16. The transformed cell of claim 15, wherein said cell is a member selected from the group consisting of a member of the genus *Streptococcus*, *E. coli*, a member of the genus *Bacillus*, a member of the genus *Pseudomonas*, a member of the genus *Salmonella*, a member of the genus *Serratia*, a yeast, a vertebrate cell, and an insect cell.

17. The transformed cell of claim 16, wherein said vertebrate cell is a member selected from the group consisting of a Chinese Hamster Ovary cell, a VERO cell, a HeLa cell, a WI38 cell, a BHK cell, a COS cell, and an MDCK cell.

18. The transformed cell of claim 16, wherein said insect cell is a cell of *Spodoptera frugiperda*.

19. The transformed prokaryotic or eukaryotic cell of claim 15, wherein said cell is capable of expressing hyaluronic acid synthase.

20. The transformed prokaryotic or eukaryotic cell of claim 15, wherein said cell is capable of producing hyaluronic acid.

21. A method of producing hyaluronic acid synthase, comprising:

(a) providing a prokaryotic or eukaryotic cell harboring a DNA sequence encoding hyaluronic acid synthase, wherein said DNA sequence is expressible in said cell;

(b) culturing said cell in a culture medium; and

(c) recovering said hyaluronic acid synthase from said cell or from said culture medium.

22. A method of producing hyaluronic acid, comprising:

(a) providing a recombinant prokaryotic or eukaryotic cell harboring a DNA sequence encoding hyaluronic acid synthase, wherein said DNA sequence is expressible in said cell;

(b) culturing said cell in a culture medium; and

(c) recovering said hyaluronic acid from said cell or from said culture medium.

23. A method of purifying hyaluronic acid synthase, comprising:

(a) culturing cells expressing a gene encoding hyaluronic acid synthase;

(b) harvesting said cells of step (a);

(c) disrupting said cells under conditions that minimize the inactivation of hyaluronic acid synthase;

(d) isolating membranes of said cells;

(e) suspending said membranes in a buffer;

(f) extracting said hyaluronic acid synthase from said membranes by adding a detergent to the membrane suspension of step (e), and then centrifuging to produce a detergent phase containing said hyaluronic acid synthase;

(g) adding polyethylene glycol to said detergent phase of step (f), thereby forming an aqueous phase containing said hyaluronic acid synthase; and

(h) recovering said hyaluronic acid synthase from said aqueous phase of step (g).

24. A method of producing hyaluronic acid of varying molecular weight, comprising:

(a) incubating UDP-GlcA and UDP-NAcGlc with hyaluronic acid synthase under conditions suitable for the synthesis of hyaluronic acid; and

(b) recovering hyaluronic acid,

wherein the molecular weight of said hyaluronic acid can be varied by varying the reaction time and/or reaction conditions under which said UDP-GlcA and said UDP-NAcGlc are incubated in the presence of said hyaluronic acid synthase.

25. Hyaluronic acid produced by the method of claim 24, wherein said hyaluronic acid is free of any contaminating proteins, pyrogenic or inflammatory substances, or viruses.

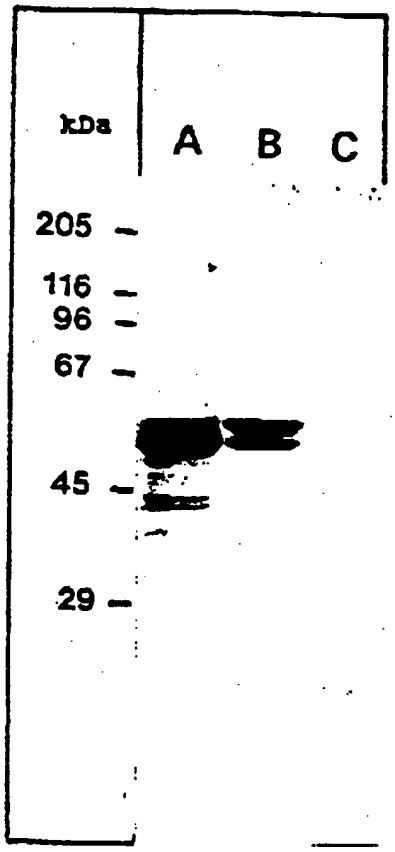

FIGURE 1

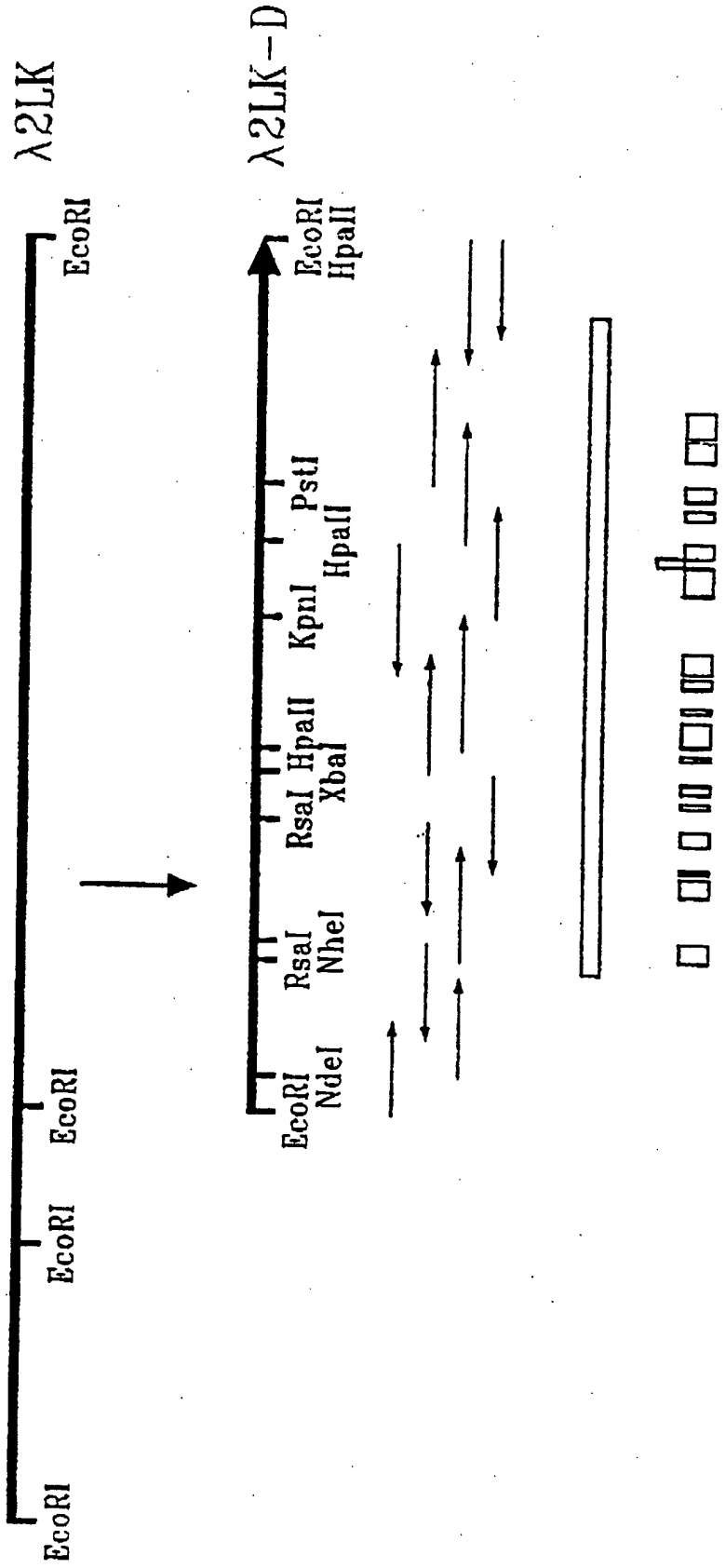
λ2LK
EcoRI
EcoRI
EcoRI
EcoRI
λ2LK-D
EcoRI
HpaII
PstI
KpnI
HpaII
RsaI
HpaII
XbaI
RsaI
NheI
EcoRI
NdeI

FIGURE 2

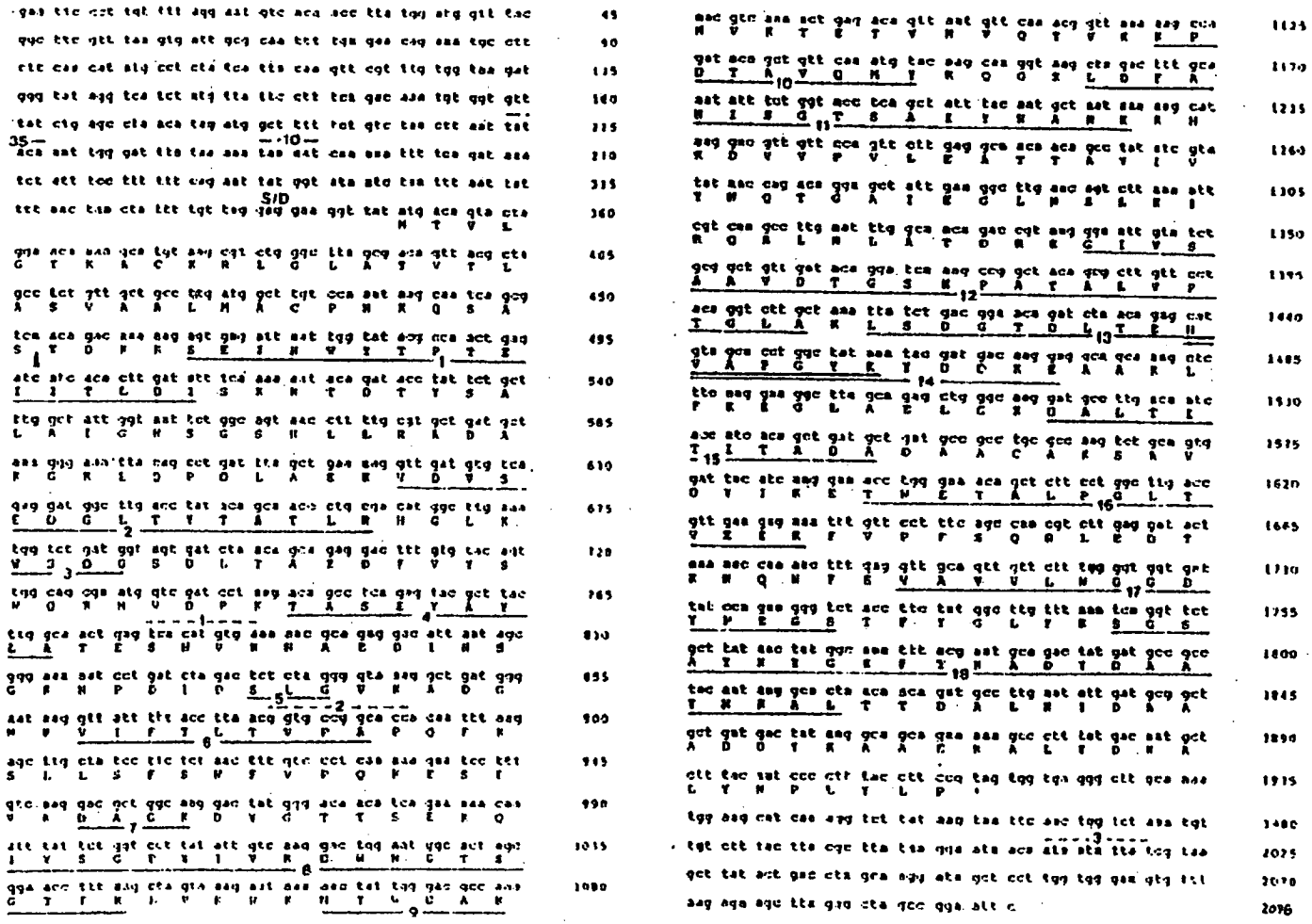

FIGURE 3

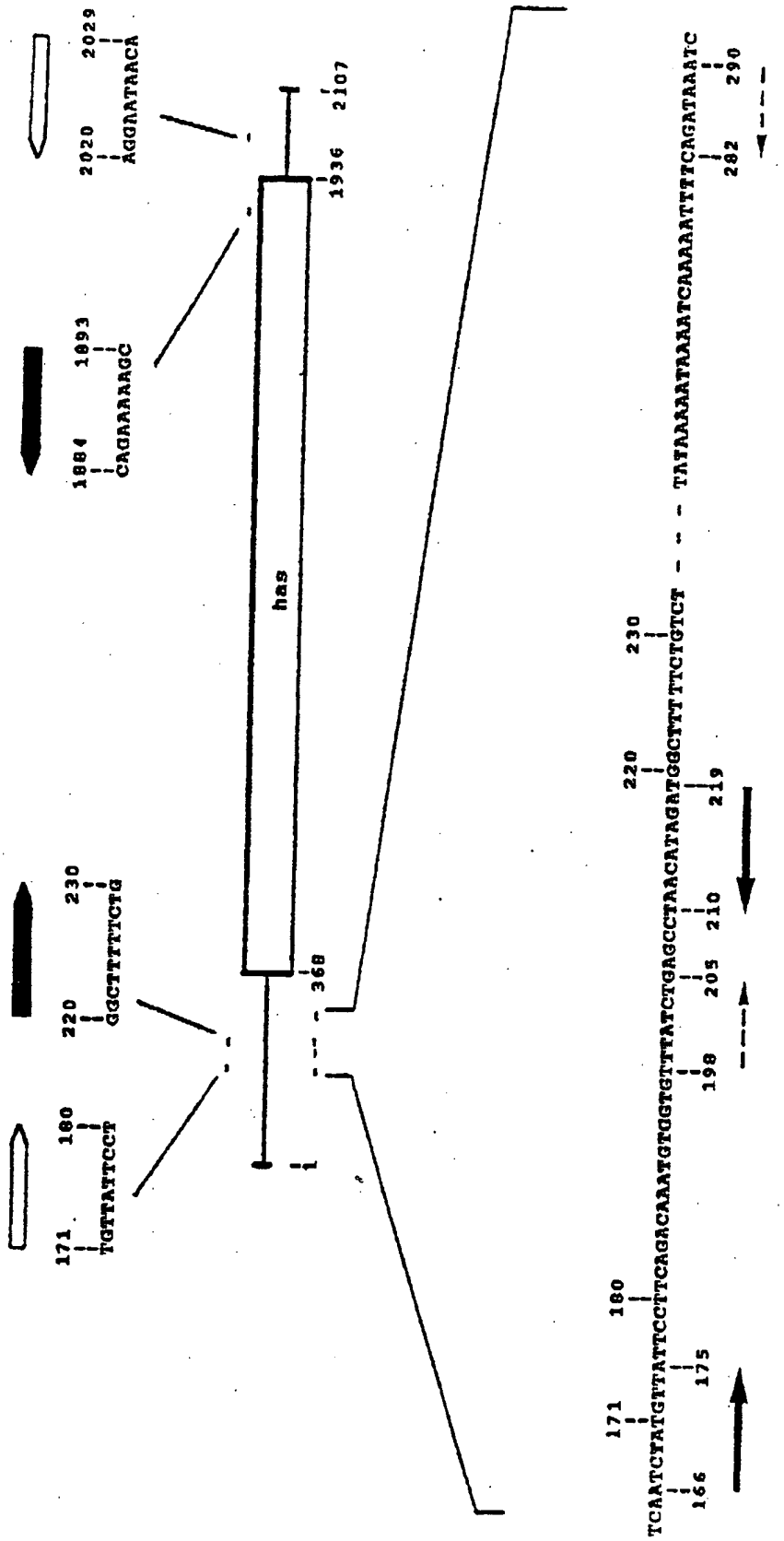
has
1
368
1884
1893
2020
2029
1936
2107
171
180
220
230
198
205
210
219
166
175
282
290
TCAATCTATGTTATTCCTTCAGACAAATGTGGTGTTTATCTGAGCCTAACATAGATGGCTTTTTCTGTCT - - - TATAAAAATAAAATCAAAAATTTTCAGATAAATC

FIGURE 4

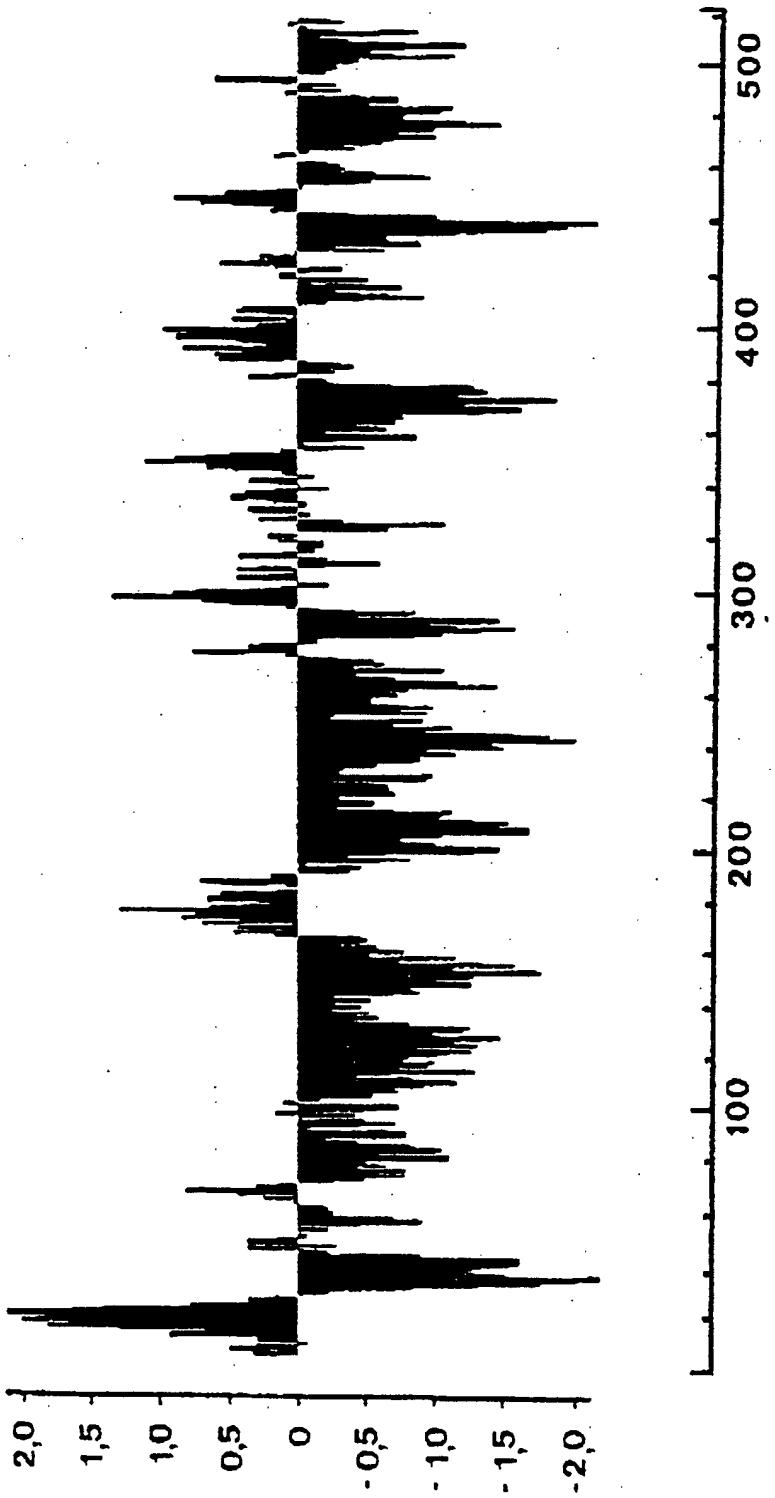
HYDROPHOB
HYDROPHIL
2,0
1,5
1,0
0,5
0
-0,5
-1,0
-1,5
-2,0
100
200
300
400
500

FIGURE 5

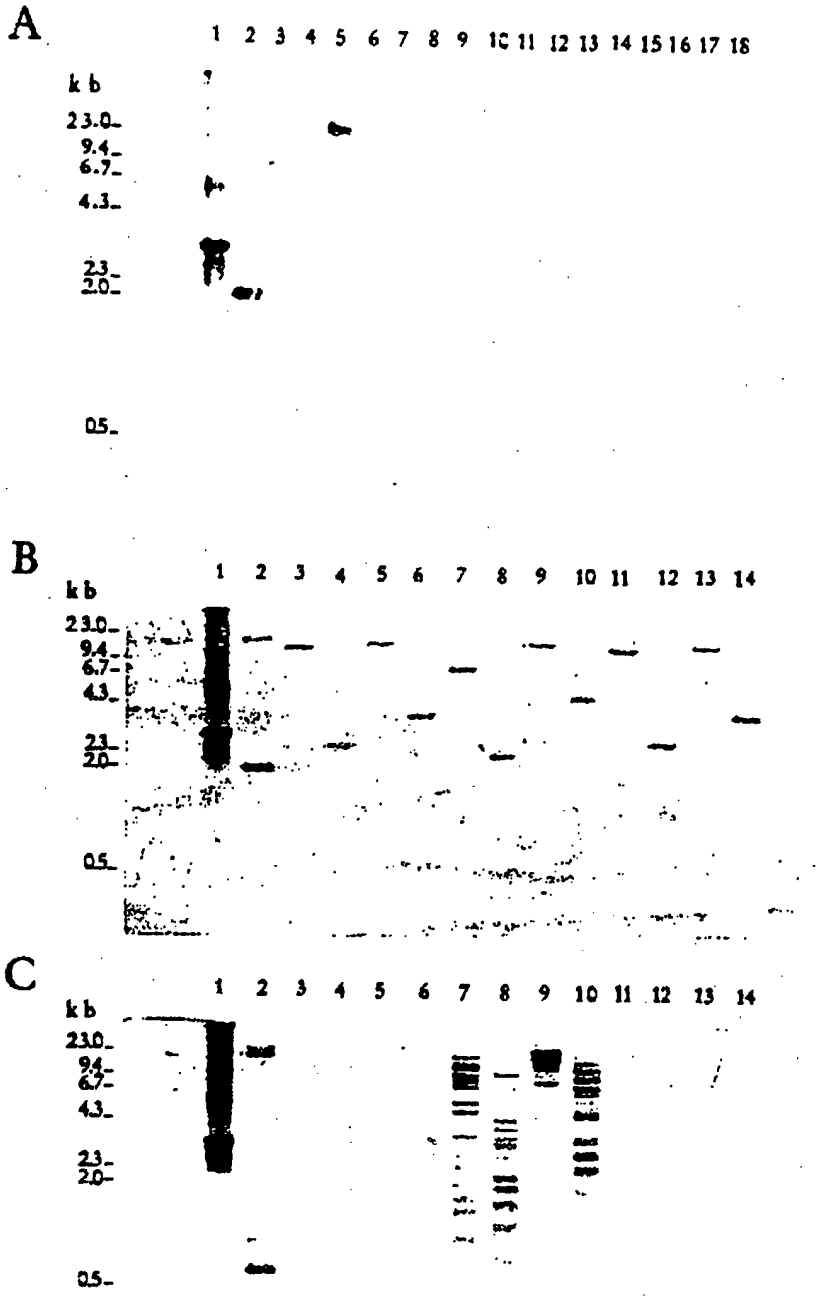
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
kb
23.0
9.4
6.7
4.3
2.3
2.0
0.5
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.0
9.4
6.7
4.3
2.3
2.0
0.5
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14
kb
23.0
9.4
6.7
4.3
2.3
2.0
0.5

FIGURE 6

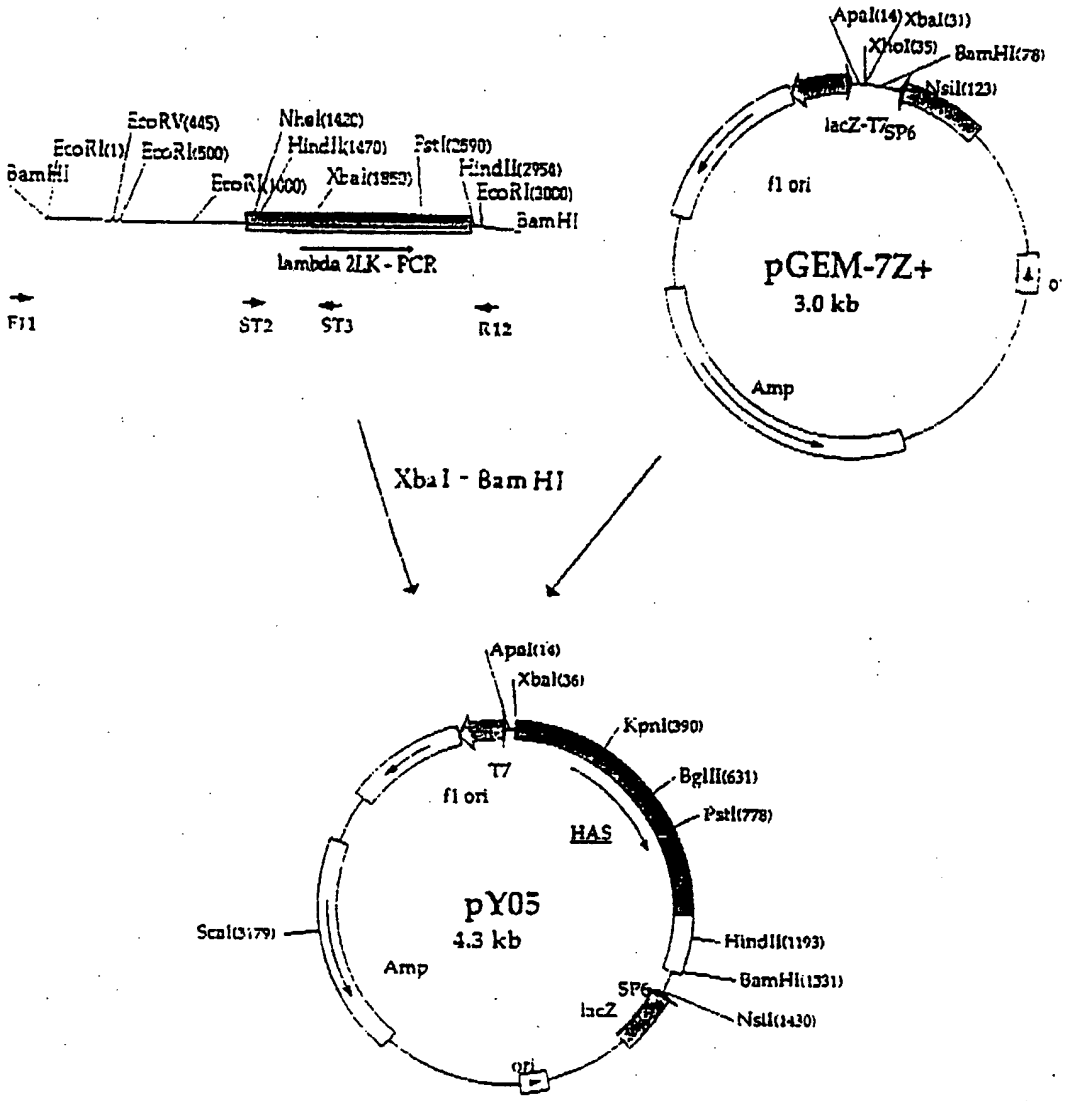
ApaI(14) XbaI(31)
XhoI(35) BamHI(78)
NsiI(123)
lacZ-T7 SP6
f1 ori
pGEM-7Z+
3.0 kb
Amp
EcoRV(445)
NheI(1420)
EcoRI(1) EcoRI(500)
HindIII(1470)
PstI(2590)
BamHI
EcoRI(1000)
XbaI(1952)
HindIII(2958)
EcoRI(3000)
BamHI
lambda 2LK - PCR
F11
ST2
ST3
R12
Xba I - Bam HI
ApaI(14)
XbaI(36)
KpnI(390)
T7
f1 ori
BglII(631)
PstI(778)
HAS
pY05
4.3 kb
ScaI(3179)
Amp
HindIII(1193)
BamHI(1331)
SP6
lacZ
NsiI(1430)
ori

FIGURE 7

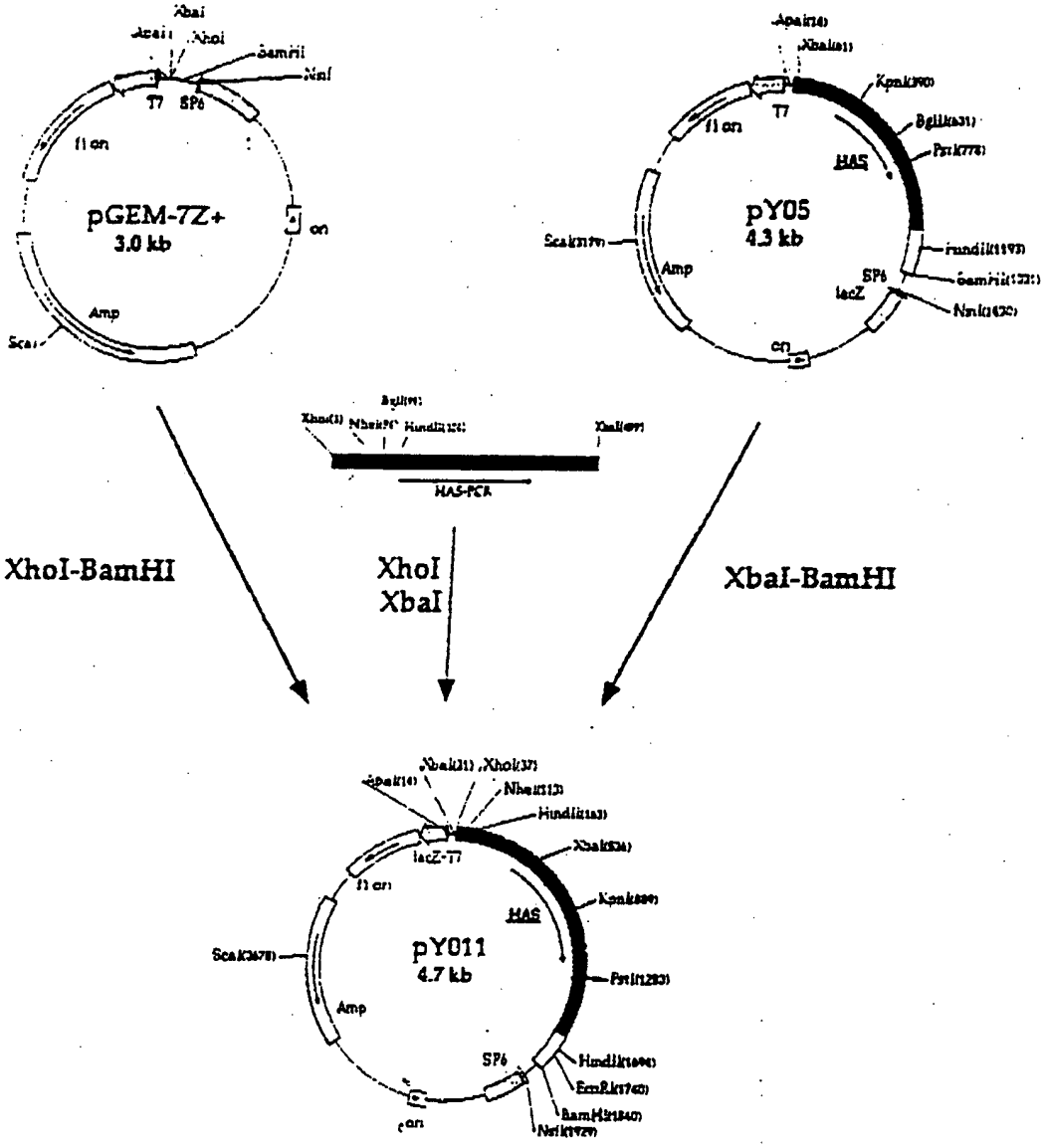
pGEM-7Z+
3.0 kb
pY05
4.3 kb
HAS-PCR
XhoI-BamHI
XhoI
XbaI
XbaI-BamHI
pY011
4.7 kb
HAS
Amp
lacZ
T7
SP6

FIGURE 8

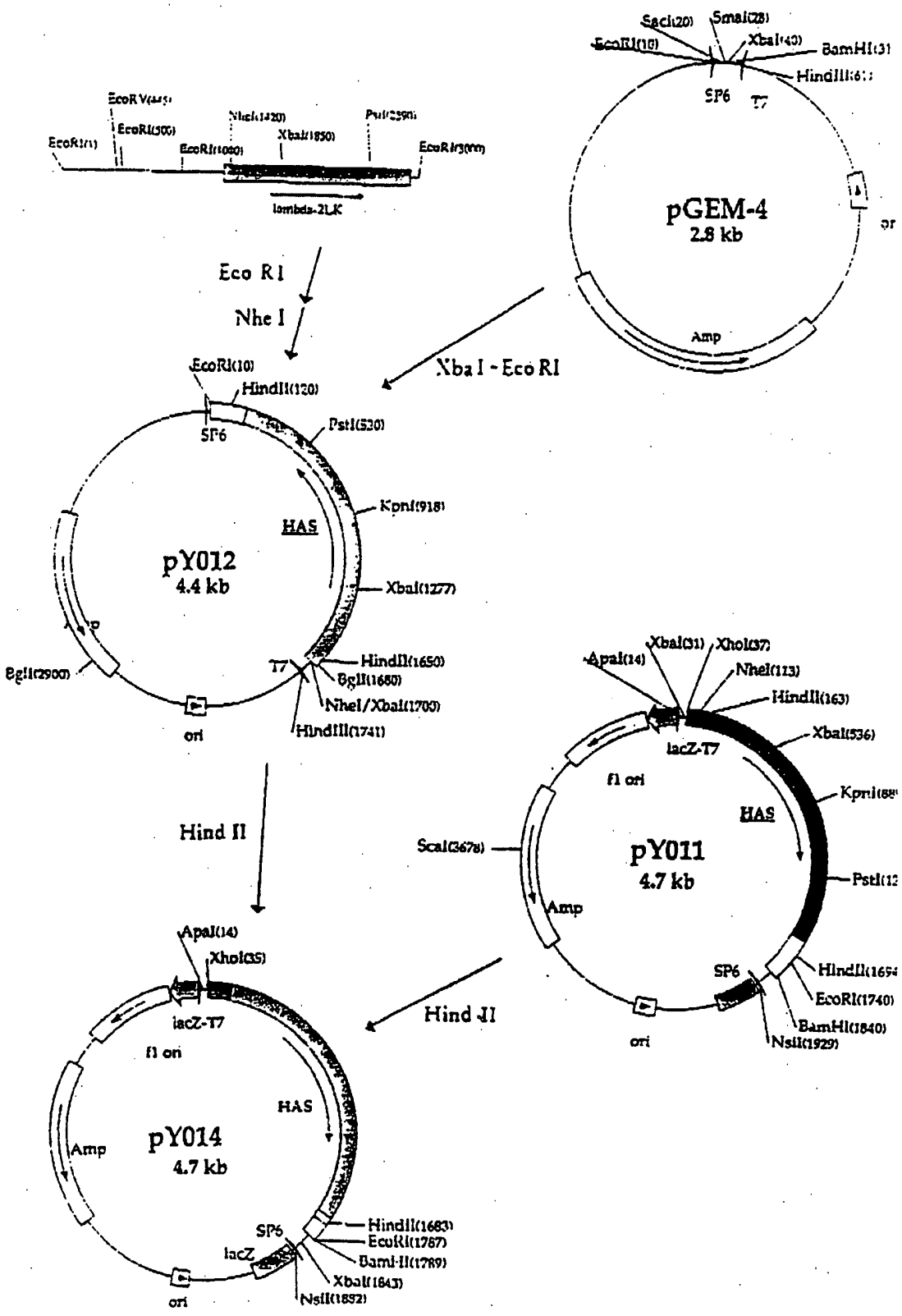
EcoR I(1)
EcoR V(445)
EcoR I(500)
Nhe I(1420)
EcoR I(1060)
Xba I(1850)
Pst I(2590)
EcoR I(3000)
lambda-2LK
Eco R I
Nhe I
SacI(20)
SmaI(28)
EcoRI(10)
XbaI(43)
BamHI(31)
HindIII(61)
SP6
T7
pGEM-4
2.8 kb
Amp
ori
Xba I - Eco RI
EcoRI(10)
HindII(120)
SP6
PstI(530)
KpnI(918)
HAS
pY012
4.4 kb
XbaI(1277)
Bgl(2900)
T7
HindII(1650)
BglI(1680)
Nhel/XbaI(1703)
HindIII(1741)
ori
Hind II
ApaI(14)
XbaI(31)
XhoI(37)
NheI(113)
HindII(163)
XbaI(536)
lacZ-T7
f1 ori
KpnI(68
HAS
ScaI(3678)
pY011
4.7 kb
PstI(12
Amp
SP6
HindII(165
EcoRI(1740)
BamHI(1840)
NsiI(1929)
ori
Hind II
ApaI(14)
XhoI(35)
lacZ-T7
f1 ori
HAS
Amp
pY014
4.7 kb
SP6
HindII(1683)
EcoRI(1787)
BamI-II(1789)
lacZ
XbaI(1843)
NsiI(1892)
ori

FIGURE 9

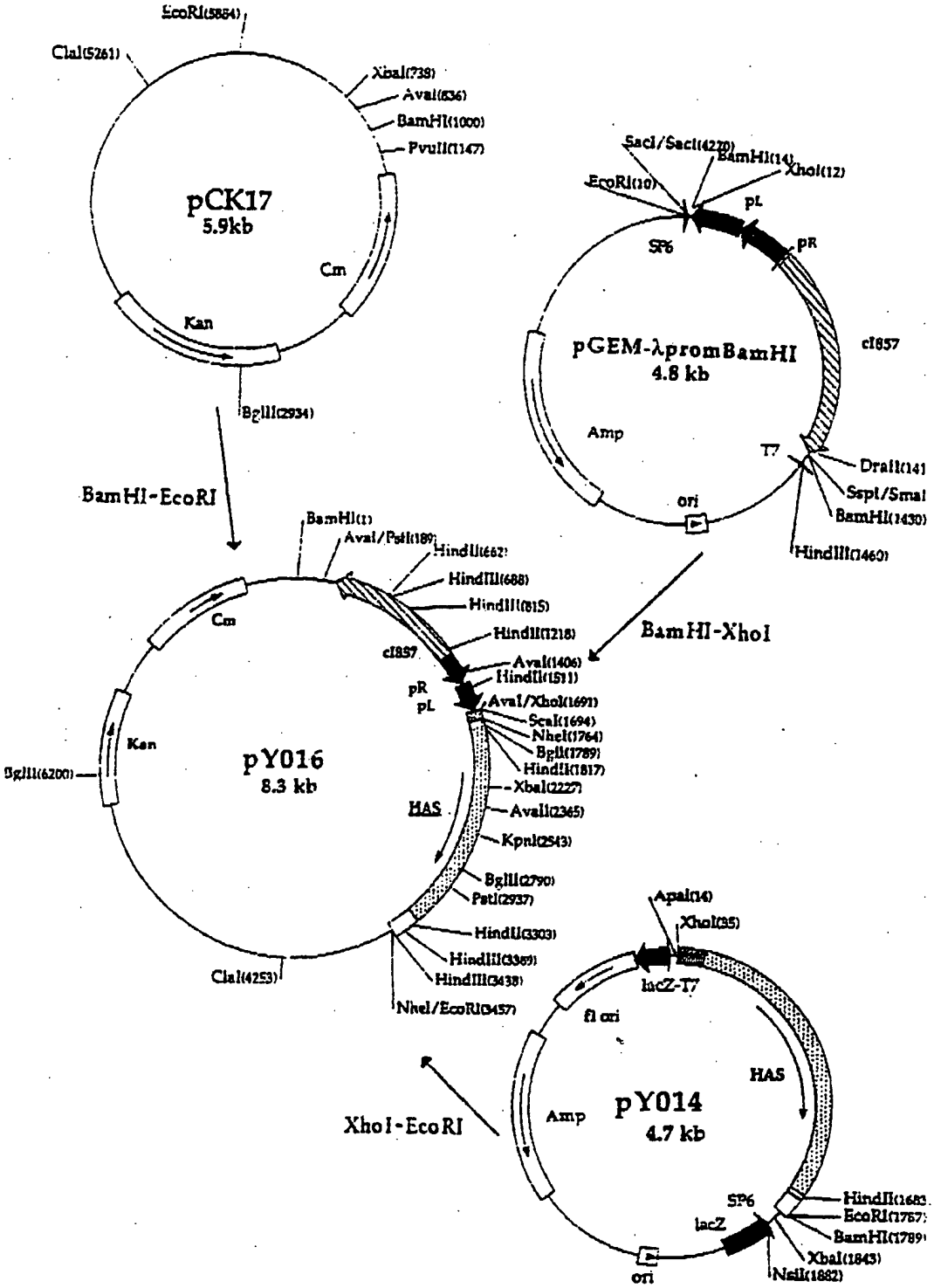
pCK17
5.9kb
EcoRI(5884)
ClaI(5261)
XbaI(738)
AvaI(836)
BamHI(1000)
PvuII(1147)
Cm
Kan
BglII(2934)
pGEM-λpromBamHI
4.8 kb
SacI/SacI(4270)
BamHI(14)
XhoI(12)
EcoRI(10)
pL
SP6
pR
cI857
Amp
T7
ori
DraII(141)
SspI/SmaI
BamHI(1430)
HindIII(1460)
BamHI-EcoRI
BamHI-XhoI
pY016
8.3 kb
BamHI(1)
AvaI/PstI(189)
HindII(662)
HindIII(688)
HindIII(815)
HindIII(1218)
cI857
AvaI(1406)
HindII(1511)
AvaI/XhoI(1691)
ScaI(1694)
NheI(1764)
BglII(1789)
HindII(1817)
XbaI(2227)
AvaII(2365)
KpnI(2543)
BglII(2790)
PstI(2937)
HindII(3303)
HindIII(3369)
HindIII(3438)
NheI/EcoRI(3457)
ClaI(4253)
BglII(6200)
HAS
pY014
4.7 kb
ApaI(14)
XhoI(35)
lacZ-T7
f1 ori
HAS
Amp
XhoI-EcoRI
SP6
lacZ
ori
HindII(1683)
EcoRI(1767)
BamHI(1789)
XbaI(1843)
NsiI(1882)

FIGURE 10

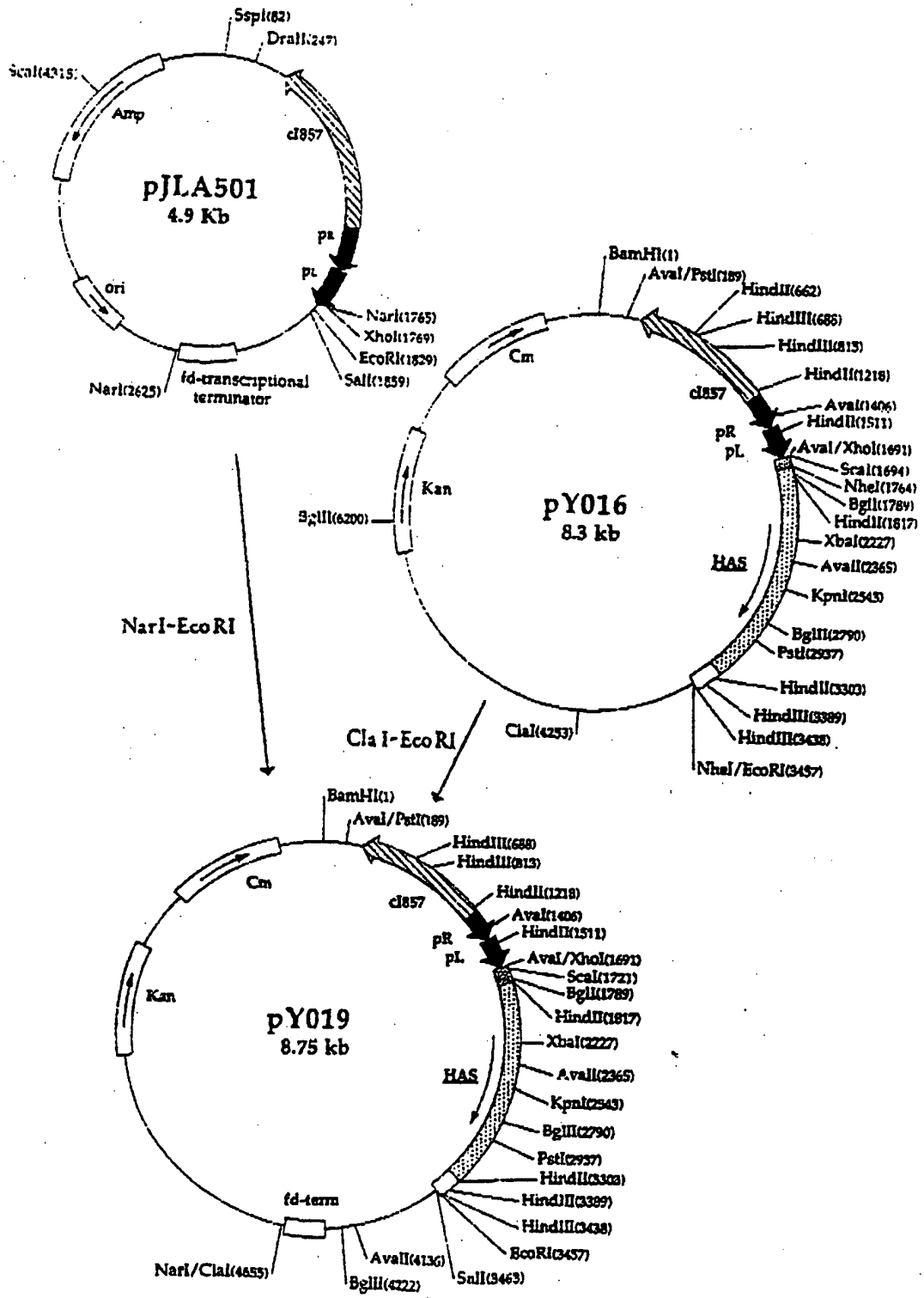
pJLA501
4.9 Kb
Amp
ori
cI857
pR
pL
fd-transcriptional terminator
pY016
8.3 kb
Kan
Cm
cI857
pR
pL
HAS
NarI-EcoRI
Cla I-EcoRI
pY019
8.75 kb
Kan
Cm
cI857
pR
pL
HAS
fd-term

FIGURE 11

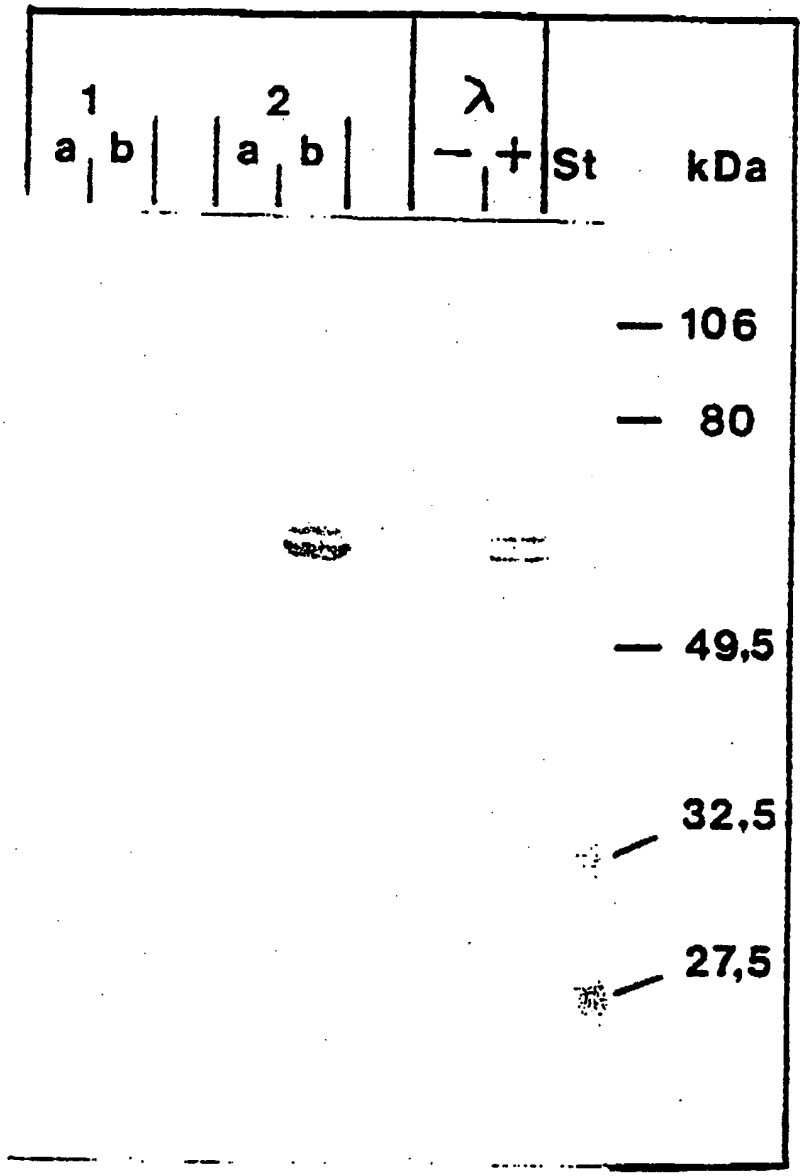

FIGURE 12

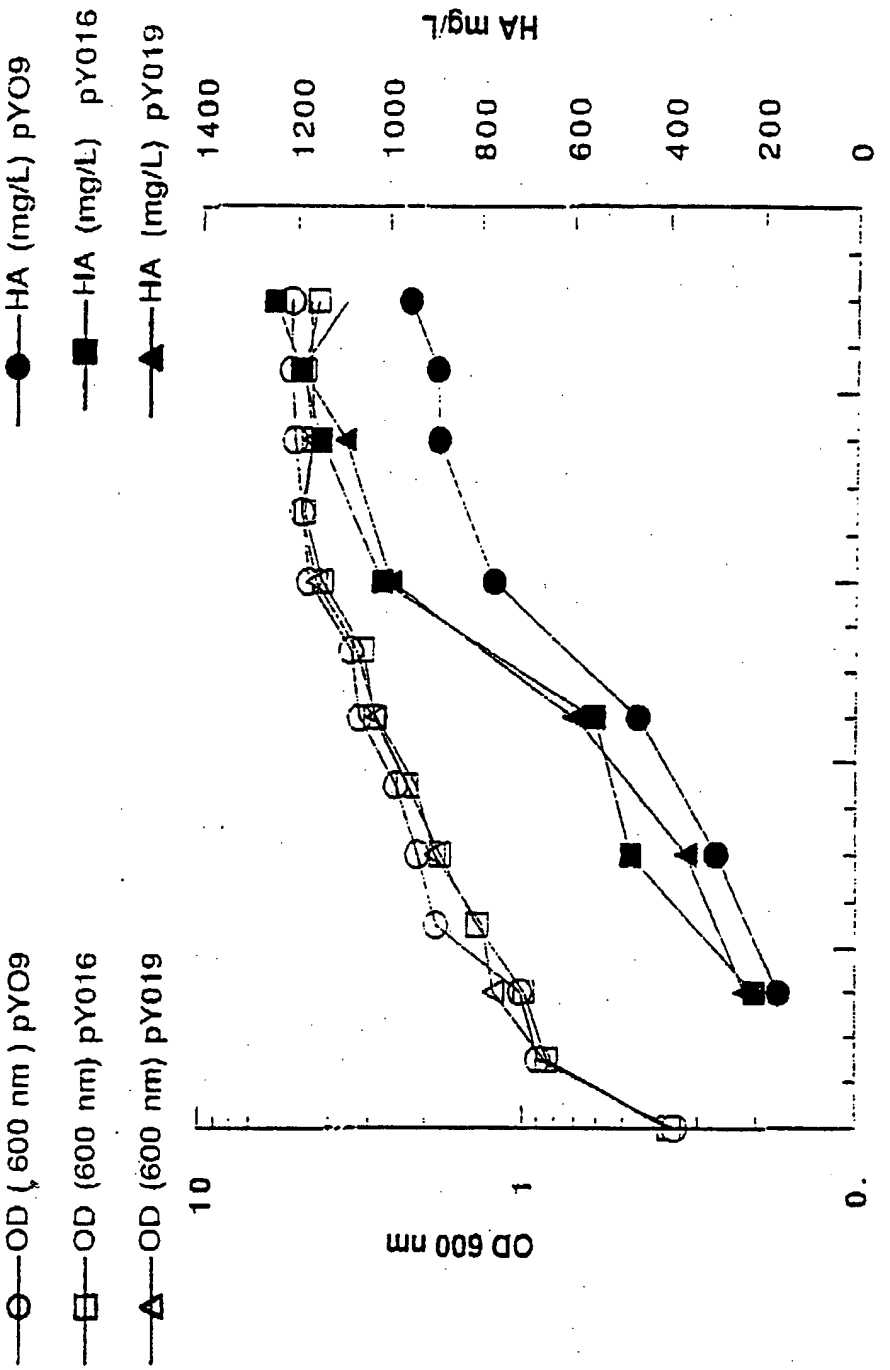
HA (mg/L) pYO9
HA (mg/L) pY016
HA (mg/L) pY019
OD (600 nm) pYO9
OD (600 nm) pY016
OD (600 nm) pY019
HA mg/L
1400
1200
1000
800
600
400
200
0
OD 600 nm
10
1
0.
2
4
6
8
10
12
time (hours)

FIGURE 13

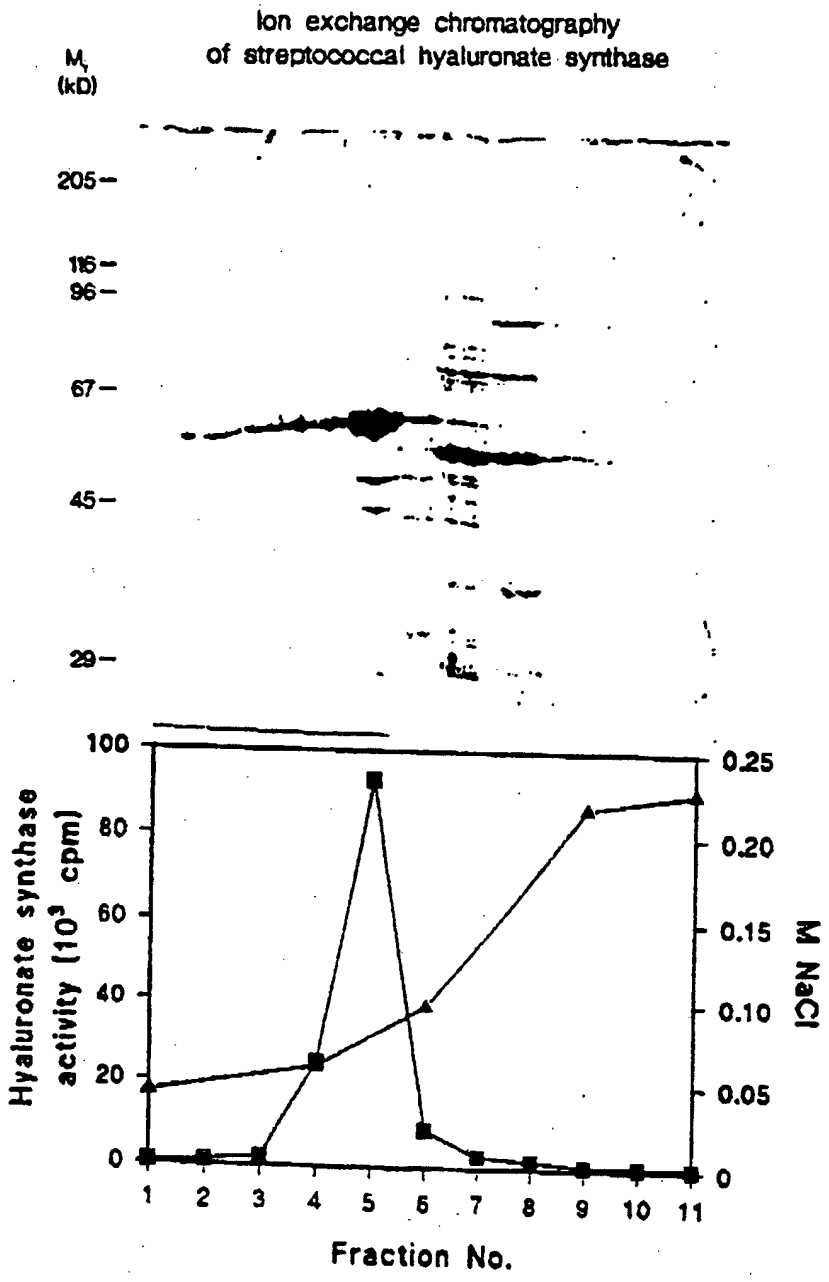
Ion exchange chromatography
of streptococcal hyaluronate synthase
M_r (kD)
205
116
96
67
45
29
Hyaluronate synthase activity (10^3 cpm)
100
80
60
40
20
0
0.25
0.20
0.15
0.10
0.05
0
M NaCl
1 2 3 4 5 6 7 8 9 10 11
Fraction No.

FIGURE 14

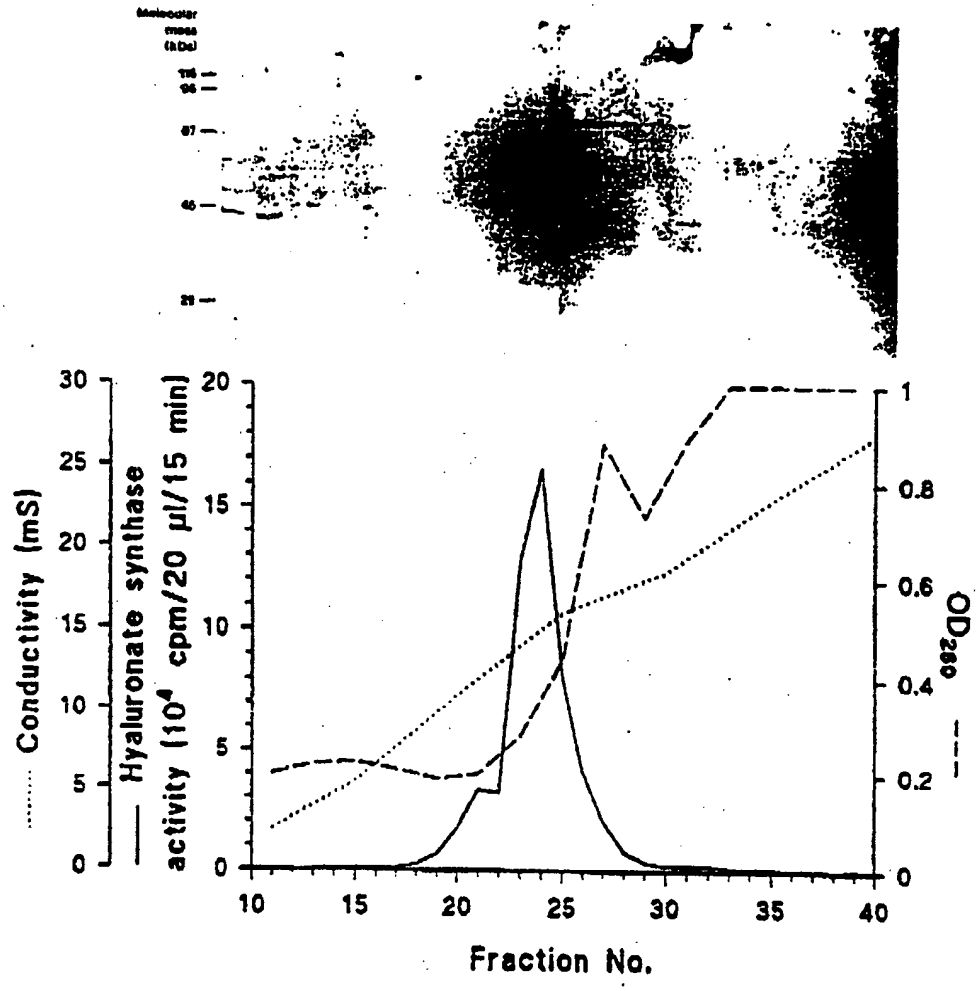
Conductivity (mS)
Hyaluronate synthase activity (10^4 cpm/20 µl/15 min)
OD_{280}
Fraction No.
0
5
10
15
20
25
30
35
40
0.2
0.4
0.6
0.8
1

FIGURE 15

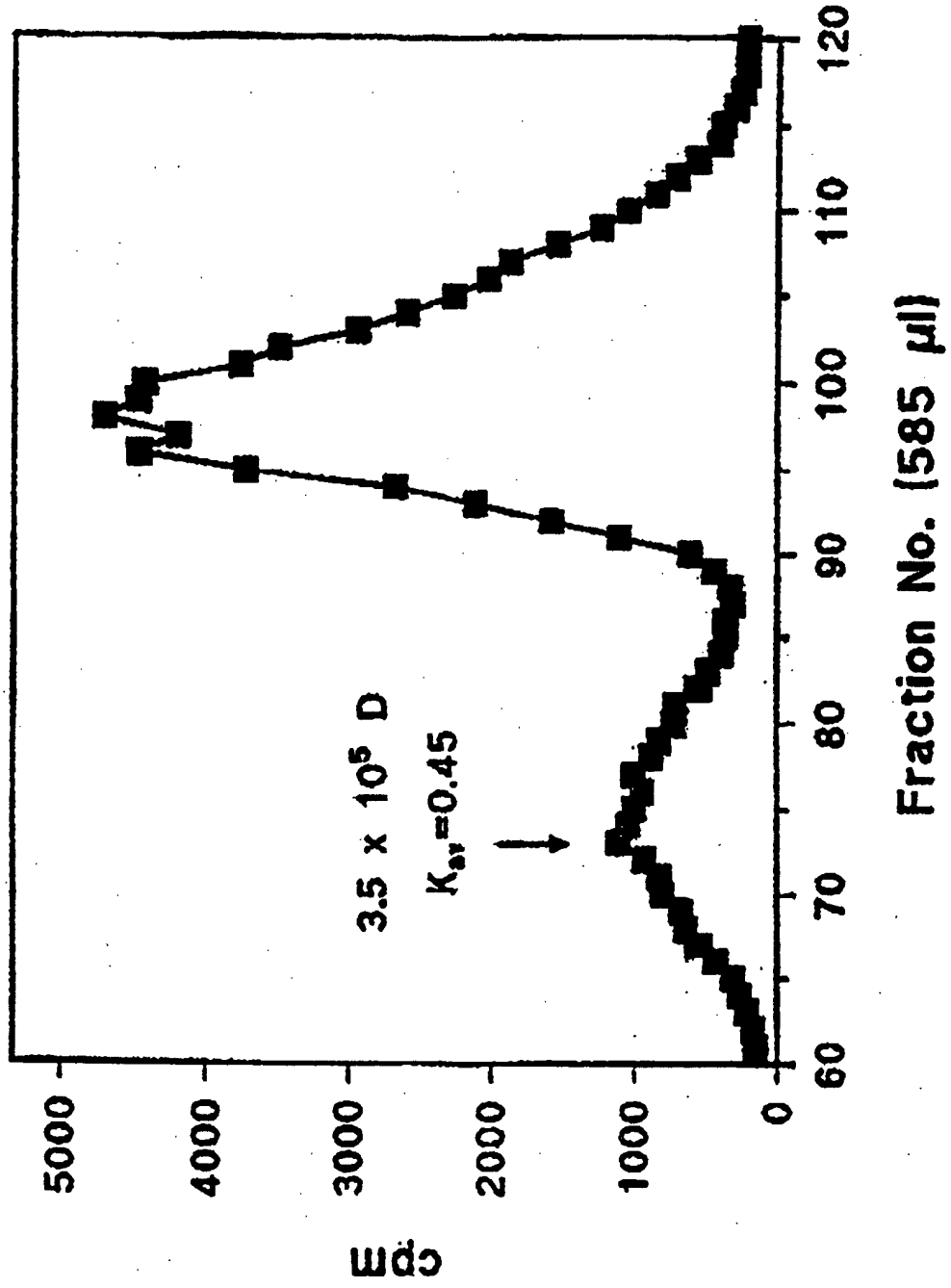
cpm
5000
4000
3000
2000
1000
0
60
70
80
90
100
110
120
Fraction No. (585 µl)
3.5 x 10⁵ D
Kav=0.45

FIGURE 16